UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

DINÂMICA DE INFECÇÃO DE *Mycoplasma hyopneumoniae* EM LEITOAS DE REPOSIÇÃO

KARINE LUDWIG TAKEUTI

PORTO ALEGRE
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DINÂMICA DE INFECÇÃO DE *Mycoplasma hyopneumoniae* EM LEITOAS DE REPOSIÇÃO

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Orientador: Dr. David Emilio Santos Neves de Barcellos
Coorientadora: Dra. Maria Pieters

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Orientador e Presidente da Comissão

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RESUMO

A infecção por *Mycoplasma (M.) hyopneumoniae* é responsável por perdas econômicas significativas na suinocultura, causando uma pneumonia crônica que normalmente afeta clinicamente suínos de crescimento e terminação. Considerando-se a importância das matrizes de menor ordem de parto na transmissão de *M. hyopneumoniae* e que uma grande quantidade de leitoas é introduzida nos planteis anualmente, este projeto teve como objetivo compreender aspectos pouco conhecidos da dinâmica de infecção de *M. hyopneumoniae* em leitoas de reposição. O primeiro estudo avaliou a dinâmica e persistência da infecção por *M. hyopneumoniae* em leitoas em condições naturais de campo. Quarenta e quatro leitoas foram selecionadas aos 20 dias de idade (ddi) e a detecção de *M. hyopneumoniae* por PCR foi avaliada mensalmente por suabe de laringe, resultando em um total de 12 coletas. Além disso, 220 leitões filhos dessas matrizes foram amostrados um dia antes do desmame para avaliação da transmissão vertical. Os resultados deste estudo demonstraram que o início da detecção ocorreu aos 110 ddi e um aumento significativo foi observado aos 140 ddi ($p<0,05$). Ao desmame, apenas 2,3% das fêmeas foi positiva e não foram detectados leitões desmamados positivos. Adicionalmente, 77,2% das leitoas foi detectada positiva por um a três meses, 4,6% por quatro a cinco meses e 18,2% nunca foi detectada positiva, indicando a presença de subpopulações de animais negativos em granjas positivas. Em um segundo estudo, avaliou-se a detecção de *M. hyopneumoniae* por PCR em leitoas de reposição interna e a variabilidade genética entre granjas foi avaliada por MLVA (*Multiple Locus Variable-number tandem repeats Analysis*). Um total de 298 leitoas provenientes de três multiplicadoras positivas foram selecionadas e coletadas uma vez para realização de ELISA e duas a três vezes para PCR em um estudo longitudinal. Ainda, a transmissão vertical foi avaliada em 425 leitões pré-desmame. Aos 150 ddi, 47 a 67,4% das leitoas foi detectada positiva por PCR, decréscimos foram observados até a última amostragem nas três granjas avaliadas ($p<0,05$), e nenhum leitão foi positivo pré-desmame. Ainda, 30,7% das leitoas não foi detectada positiva em nenhuma nas amostragens e os resultados de MLVA indicaram um ampla variabilidade genética de *M. hyopneumoniae* em leitoas aos 150 ddi. No

terceiro estudo, 210 leitoas de reposição negativas para *M. hyopneumoniae* introduzidas em três granjas positivas foram selecionadas e avaliadas longitudinalmente por PCR e ELISA com o objetivo de comparar dois tipos de fluxo de aclimatação (*all-in all-out* ou fluxo contínuo). Ainda, a variabilidade genética foi analisada por MLVA. Observou-se um aumento significativo ($p<0,05$) nas prevalências de leitoas positivas para *M. hyopneumoniae* por PCR na segunda coleta e um decréscimo ao longo do tempo independentemente do tipo de fluxo de aclimatação utilizado. Os resultados de ELISA revelaram que as três granjas avaliadas tiveram um aumento significativo na prevalência de leitoas positivas da primeira para a segunda coleta ($p<0,05$), que se manteve alta até o fim do experimento. Ainda, baixa variabilidade genética de *M. hyopneumoniae* foi observada por MLVA. Um quarto estudo também foi conduzido com o objetivo de avaliar a absorção e detecção de *M. hyopneumoniae* utilizando-se suabes de nylon flocados e de ponta de rayon. Os resultados deste experimento indicaram uma maior absorção e uma maior detecção de *M. hyopneumoniae* por PCR ($p<0,05$) utilizando-se suabes de nylon flocados.

Palavras-chave: diagnóstico, epidemiologia, variabilidade genética.

ABSTRACT

Mycoplasma (M.) hyopneumoniae infection is responsible for important economic losses to pig production, causing a chronic pneumonia that usually affects growing and finishing pigs. Regarding the importance of low parity dams on *M. hyopneumoniae* transmission and that a high proportion of gilts is introduced in the farms every year, this project aimed to better understand unknown aspects about the infection dynamics of *M. hyopneumoniae* in replacement gilts. The first study assessed the dynamics and persistence of *M. hyopneumoniae* infection in gilts in natural conditions. Forty-four gilts were selected at 20 days of age (doa) and *M. hyopneumoniae* detection was evaluated using laryngeal swabs for PCR testing every month, resulting in 12 samplings. Additionally, 220 piglets born from the selected dams were sampled one day prior to weaning to evaluate vertical transmission. The results of this study showed that the first detection occurred at 110 doa and a significant increase was observed at 140 doa ($p<0.05$). A small proportion (2.3%) of positive gilts was detected one day prior to weaning and no piglets were detected positive at the same sampling moment. Moreover, 77.2% of the gilts was detected positive for one to three months, 4.6% for four to five months, and a lack of detection was observed in 18.2% of the gilts, indicating the presence of negative subpopulations in positive farms. A second study assessed the *M. hyopneumoniae* detection in self-replacement gilts longitudinally. A total of 298 gilts from three positive multiplier farms were selected and sampled once for ELISA testing and two or three times for PCR. Moreover, vertical transmission was evaluated in 425 piglets one day prior to weaning, and the genetic variability within farms was assessed in gilts by MLVA (*Multiple Locus Variable-number tandem repeats Analysis*). The prevalence of positive gilts at 150 doa ranged from 47 to 67.4% within farms by PCR, decreases were observed up to the last sampling in all farms ($p<0.05$), and no positive piglets were detected prior to weaning. A lack of detection was observed in 30.7% of the gilts during the study, and high genetic variability was detected within farms. In the third study, 210 *M. hyopneumoniae* negative purchased gilts were introduced in three farms with two types of acclimation flow (all-in all-out or continuous flow). The gilts were selected for ELISA and PCR testing in a longitudinal study, which

aimed to compare the infection dynamics regarding the type of flow. Also, genetic variability was assessed by MLVA. The results showed a significant increase ($p<0.05$) in the prevalence of *M. hyopneumoniae* positive gilts by PCR in the second sampling, and a decrease over time regardless the type of flow. The ELISA results revealed a significant increase in the prevalence of positive gilts in the three farms ($p<0.05$) from the first to the second sampling, which remained high up to the completion of the study. Moreover, limited genetic variability of *M. hyopneumoniae* was observed by MLVA testing. A fourth study was also performed, which aimed to assess the absorption and detection of *M. hyopneumoniae* using two types of swabs (nylon-flocked and rayon-bud). The results indicated a higher absorption and *M. hyopneumoniae* detection by PCR using nylon-flocked swabs ($p<0.05$).

Keywords: diagnostic, epidemiology, genetic variability.

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1. INTRODUÇÃO

Atualmente, o Brasil é o quarto maior produtor e exportador de carne suína do mundo (ABPA, 2015), e o Rio Grande do Sul, Santa Catarina, Paraná e Minas Gerais são os estados que concentram a maior parte do plantel, sendo a região Sul responsável por 49% do rebanho brasileiro e por mais de 66% de todo o abate nacional (IBGE, 2015). A forte participação do Brasil no mercado internacional de carne suína se deve, em parte, ao desenvolvimento de tecnologias que visam a melhoria da sanidade, nutrição, reprodução, ambiência e manejo. Embora tenha ocorrido avanço sanitário significativo na suinocultura mundial, o aumento na concentração de animais por instalações, bem como o aumento de granjas por regiões favoreceu o surgimento de novas doenças e a persistência da maioria das já existentes (BARCELLOS et al., 2008).

As pneumonias e as infecções gastrintestinais representam os principais desafios sanitários da suinocultura do nosso país. Entre todos os agentes causadores de pneumonia, *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*), causador da pneumonia enzoótica ou micoplásmica, é considerado pela maioria dos clínicos e pesquisadores brasileiros como o patógeno mais importante para suínos (BARCELLOS, 2017). Trabalhos realizados em frigoríficos brasileiros mostram que suínos na idade de abate apresentam entre 29,6% (STEPAN, 1995) e 75,7% (SILVA et al., 2001) dos pulmões com lesões sugestivas de pneumonia, e grande parte delas se deve à infecção por *M. hyopneumoniae*. Trata-se de uma pneumonia crônica que afeta suínos de todas as idades, principalmente nas fases de crescimento e terminação (DOS SANTOS et al., 2012). Além dos efeitos diretos da doença, o agente predispõe os animais a infecções secundárias por vírus ou outras bactérias. Essa interação mista entre dois ou mais patógenos é denominada “Complexo de Doenças Respiratórias dos Suínos” (CDRS), que normalmente agrava o quadro clínico dos animais afetados, aumenta as taxas de mortalidade e reduz a *performance* (NEUMANN et al., 2009; KICH et al., 2010; DOS SANTOS et al., 2012; THACKER & MINION, 2012).

A transmissão de *M. hyopneumoniae* ocorre via vertical, da porca para os leitões durante o período lactacional (CALSAMIGLIA & PIJOAN, 2000), ou horizontal, de leitão para leitão (FANO et al., 2005), além da transmissão pelo ar que pode ocorrer por mais de 9 km de distância (OTAKE et al., 2010). Diversos trabalhos sobre a dinâmica de infecção por *M. hyopneumoniae* foram realizados (SIBILA et al., 2004; FANO et al., 2005; FANO et al., 2006ab; FANO et al., 2007; PIETERS et al., 2009), demonstrando o papel das leitoas na transmissão de *M. hyopneumoniae* para suas leitegadas e os prejuízos sanitários e econômicos em decorrência da infecção precoce. A fácil transmissão do patógeno, associada à alta prevalência na suinocultura mundial, dificulta a manutenção de rebanhos livres de *M. hyopneumoniae*. Por este motivo, a erradicação do agente dos planteis não ocorre normalmente e se faz necessário conviver com a infecção pelo agente em níveis economicamente aceitáveis através da adoção de medidas eficientes de prevenção e controle (DOS SANTOS et al., 2012).

2. CAPÍTULO I - REVISÃO BIBLIOGRÁFICA

2.1. Pneumonias em suínos

No sistema tecnificado de produção de suínos, a maior concentração de animais por galpão/baia e de granjas por região favorece a disseminação e instalação de doenças respiratórias (BARCELLOS et al., 2008) e as infecções por *M. hyopneumoniae* ocorrem continuamente (FANO et al., 2007). Além disso, o suíno é particularmente suscetível a pneumonias devido à anatomia pulmonar, como a presença de poucos bronquíolos, pleura espessa e lobos bem definidos, dificultando a eliminação de partículas (LÓPEZ, 2007).

A ocorrência das pneumonias em suínos dependem de múltiplos fatores, como hospedeiro, agente infeccioso, condições ambientais e de manejo (LÓPEZ, 2007; MORES, 2010), de forma que a alta densidade animal, mistura de animais de diferentes origens, aquecimento deficiente, ventilação e umidade inadequadas são alguns dos fatores que podem predispor à ocorrência de doenças respiratórias (MAES et al., 2008). Dados de prevalência de lesões pulmonares em suínos podem ser obtidos principalmente através de monitorias de frigorífico. Embora sejam feitas com frequência, poucas informações são encontradas na literatura. Dados publicados informam que as pneumonias ocorrem de forma endêmica no Brasil, e que esses percentuais oscilam (Tabela 1), podendo atingir até 75,7% (SILVA et al., 2001) dos pulmões avaliados.

Diversos agentes podem causar pneumonias em suínos. Geralmente agentes de pneumonias virais, como o Vírus da Influenza Suína (IAV), Circovirus Suíno Tipo 2 (PCV2), Coronavirus Respiratório (PRCV) e Vírus da Síndrome Reprodutiva e Respiratória dos Suínos (PRRSV), predispõem à ocorrência de infecções bacterianas secundárias, aumentando a severidade das pneumonias (MORES, 2010), o que caracteriza um sinergismo viral-bacteriano (LÓPEZ, 2007). *Mycoplasma hyopneumoniae* possui essa mesma característica e é capaz de causar imunossupressão ao animal, predispondo-o a coinfecções com vírus ou outras bactérias (NEUMANN et al., 2009; KICH et

al., 2010; DOS SANTOS et al., 2012; THACKER & MINION, 2012). No Brasil, a infecção mista por *M. hyopneumoniae* e *Pasteurella (P). multocida* tipo A é frequentemente observada (BARCELLOS et al., 2008; KICH et al., 2010; TAKEUTI et al., 2013), além do envolvimento também comum com *Actinobacillus (A.) pleuropneumoniae* e IAV (MORES, 2006; SCHWARTZ, 2016).

Tabela 1. Prevalências médias de lesões pulmonares em suínos ao abate no Brasil.

Autores	**Regiões avaliadas**	**Prevalência de lesões pulmonares ao abate**
Sobestiansky et al., 1987	Sul	55,3%
Reis et al., 1992	Sudeste	53,9%
Stepan, 1995	Sul	29,6%
Sobestiansky et al., 1999	Sul	42,6%
Sobestiansky et al., 2001	Sul	54,9%
Silva et al., 2001	Sul, Sudeste e Centro-oeste	75,7%
Silva et al., 2002	Sul, Sudeste e Centro-oeste	69,25
Silva et al., 2006	Sul, Sudeste e Centro-oeste	63,6%
Moreira et al., 2011	Centro-oeste	53,6%

A pneumonia causada por *M. hyopneumoniae* está presente em todos os países onde a suinocultura é importante (DESROSIERS, 2001). Nos Estados Unidos da América (EUA), acredita-se que 52,7% das creches e 68% das terminações de suínos tenham problemas associados ao *M. hyopneumoniae* (THACKER & MINION, 2012). No Brasil, mais de 95% das granjas comerciais são positivas para *M. hyopneumoniae* e a ocorrência ou

severidade da doença está totalmente relacionada a condições ambientais, de manejo e alojamento. Embora os estudos de prevalência de pneumonia em abates de suínos nem sempre revelem os agentes causais, acredita-se que *M. hyopneumoniae* seja o principal, promovendo uma porta de entrada para outros agentes infecciosos (BARCELLOS, 2017).

As pneumonias determinam alto impacto econômico para a suinocultura através da mortalidade de animais, gastos com medicamentos e vacinas, piora na conversão alimentar, redução na ingestão de alimento e de ganho de peso diário (THACKER & MINION, 2012). Planteis positivos para *M. hyopneumoniae* apresentam 1,4% a mais de mortalidade de suínos de terminação; 1,0% a mais de descartes, além de US$ 0,26 a mais de gastos por animal com tratamentos quando comparados a granjas livres (YESKE, 2014). Ainda, estima-se que a cada aumento de 10% de área pulmonar afetada, há um decréscimo médio de 41g no ganho de peso diário e aumento de 16,7 dias para os animais acometidos atingirem o peso de abate (HILL et al., 1992).

2.2. *Mycoplasma hyopneumoniae*

2.2.1. Etiologia

Os micoplasmas pertencem à classe dos Mollicutes e se caracterizam pelo tamanho (0,2 a 0,3µm) e genoma pequeno (580 a 1380kb), cerca de cinco vezes menor que a média das bactérias (RAZIN et al., 1998). A ausência de genes que sintetizam aminoácidos e ácidos graxos resulta na necessidade dessas bactérias em obter nutrientes de seus hospedeiros (HUTCHISON & MONTAGUE, 2002). Essa característica também influencia no diagnóstico bacteriológico, tornando-as de difícil isolamento (BROWNING et al., 2010). Não apresentam a parede celular externa e tem como limite externo uma membrana plasmática composta por proteínas, glicoproteínas, glicolipídeos e fosfolipídeos, tornando-as pleomórficas e suscetíveis à ação de desinfetantes e à dessecação. A ausência de síntese de peptideoglicano impossibilita a utilização de coloração de Gram. Dessa forma, os micoplasmas são corados apenas com

colorações do tipo Romanowsky, como o Giemsa (BROWNING et al., 2010; MARKEY et al., 2013).

Variabilidade genética e da patogenicidade têm sido observadas em cepas de *M. hyopneumoniae* (THACKER & MINION, 2012). O sequenciamento completo do genoma da bactéria (cepa 232) revelou a presença de pelo menos 22 regiões que se repetem (VNTRs – *variable number of tandem nucleotide repeats*), presentes em 12 genes que codificam proteínas de superfície (MINION et al., 2004), como a P97 e P146. Essas repetições são locais ativos para recombinação genética (ROCHA & BLANCHARD, 2002), que podem ser detectadas através de MLVA (*Multiple locus variable number tandem repeats analysis*; VRANCKX et al., 2011; DOS SANTOS et al., 2015; PANTOJA et al., 2016; MICHIELS et al., 2017). Já as diferenças de virulência foram observadas por Vicca et al. (2003) após inoculações experimentais em animais livres de *M. hyopneumoniae*. Os autores observaram diferentes graus de patogenicidade entre cepas, sendo que as mais virulentas apresentaram maior capacidade de colonizar ou aderir ao epitélio ciliado, se multiplicando mais rapidamente e causando sinais clínicos e lesões macro e microscópicas mais severas. Ainda, Villarreal et al. (2009) observaram que suínos inoculados com cepas de baixa virulência e desafiados com cepas de alta patogenicidade 4 semanas após não estavam protegidos frente à cepa de alta virulência, ou seja, reinfecções podem ocorrer quando um animal é infectado com cepas de patogenicidade distintas.

Além do *M. hyopneumoniae*, outros micoplasmas são patogênicos aos suínos. *M. hyosynoviae* é responsável por causar sinovites e artrites esporádicas em animais em crescimento (10 a 30 semanas de idade) e *M. hyorhinis* causa poliserosites fibrinosas e artrites em leitões de 3 a 10 semanas de idade (NEUMANN et al., 2009; THACKER & MINION, 2012). O papel do *M. hyorhinis* como agente primário de pneumonias em suínos vem sendo estudado. Em inoculações experimentais, Lin et al. (2006) observaram que *M. hyorhinis* foi capaz de produzir lesões macro e microscópicas semelhantes àquelas causadas pela pneumonia por *M. hyopneumoniae.* Além disso, *M. suis* também pode afetar suínos, infectando eritrócitos, resultando em um quadro de anemia infecciosa (THACKER & MINION, 2012).

2.2.2. Patogenia

A patogenia do *M. hyopneumoniae* é complexa e se caracteriza pela colonização prolongada do epitélio respiratório, aumento da resposta inflamatória, supressão dos mecanismos de defesa do trato respiratório e interação do agente com outros patógenos (THACKER & MINION, 2012).

Através de adesinas, como a P97 e a P146, o agente se adere às células epiteliais ciliadas da traqueia e brônquios, colonizando o trato respiratório, causando ciliostase e destruição dos cílios (BLANCHARD et al., 1992) através do aumento de cálcio intracelular no epitélio ciliado (PARK et al., 2002). A aderência do *M. hyopneumoniae* aos cílios do trato respiratório permite que a bactéria escape do sistema imune do hospedeiro, devido à dificuldade de acesso dos mediadores de defesa nessa região (como células inflamatórias, citocinas e anticorpos), o que torna o curso clínico longo (DOS SANTOS et al., 2012). A hiperplasia de BALT (tecido linfoide associado aos brônquios) é observada em decorrência do estímulo do *M. hyopneumoniae*, ocorrendo aumento da produção de citocinas pró-inflamatórias (IL-1α, IL-1β, IL-2, IL-8, IL-10, TNF-α e lFN-γ) pelos macrófagos e linfócitos (REDONDO et al., 2009).

A colonização do epitélio respiratório, associada ao aumento da resposta inflamatória no pulmão reduz a eficácia das defesas do trato respiratório, predispondo o animal a infecções por patógenos oportunistas (BROWNING et al., 2010; DOS SANTOS et al., 2012). A infecção do *M. hyopneumoniae* com *P. multocida* tipo A, *A. pleuropneumoniae*, *Streptococcus suis*, *Haemophilus* (*H.*) *parasuis*, IAV, PCV2 ou PRRSV (em países endêmicos) caracteriza o CDRS (FANO et al., 2005; KICH et al., 2010; DOS SANTOS et al., 2012; THACKER & MINION, 2012; SCHWARTZ, 2016). No Brasil, destacam-se as infecções secundárias causadas pela *P. multocida* tipo A. Após avaliação histológica em pulmões de suínos ao abate, Takeuti et al. (2013) observaram que 97,6% das amostras de pulmão avaliadas apresentavam lesões compatíveis com a coinfecção entre *P. multocida* e *M. hyopneumoniae*. Essa interação é frequentemente observada, pois *M. hyopneumoniae* lesiona os cílios (AMASS et al., 1994) e modifica a composição de glicoconjugados presentes nas células

epiteliais de brônquios e bronquíolos, aumentando a afinidade da *P. multocida* tipo A com esses receptores (PARK et al., 2016b). Além de predispor suínos a infecções secundárias, *M. hyopneumoniae* pode atuar em sinergismo com outros agentes infecciosos. Suínos infectados por *M. hyopneumoniae* e desafiados com IAV após um período de 21 dias apresentam sinais clínicos e lesões pulmonares mais graves e redução de ganho de peso diário mais acentuada quando comparados a suínos inoculados apenas com o vírus (DEBLANC et al., 2012). Sinais clínicos respiratórios mais graves também foram observados em infecções mistas entre esses dois agentes, devido à uma resposta inflamatória mais acentuada com aumento da produção de macrófagos, neutrófilos e citocinas pró-inflamatórias, como IL-6, IL-1β e TNF-α (DEBLANC et al., 2016).

O número médio de infecções secundárias causadas por uma infecção primária, ou seja, a taxa média de reprodução ajustada da infecção (Rn) por *M. hyopneumoniae* é baixa. Em experimento realizado por Meyns et al. (2004) foi obtido um Rn médio de 1,16 em um período de 6 semanas, ou seja, um leitão infectado é capaz de infectar aproximadamente um suscetível nesse período. Embora a capacidade infectante do *M. hyopneumoniae* seja baixa, é suficiente para manter a infecção nos planteis. Dessa forma, é esperado que se encontrem animais positivos durante toda a fase de terminação, onde animais infectados atuam como potenciais transmissores da infecção para suínos suscetíveis. Variações nas taxas de transmissão podem existir de acordo com a patogenicidade da cepa. Em condições experimentais, houve variação no Rn entre 0,33 (cepas pouco patogênicas) e 5,38 (cepas muito virulentas). Variantes de alta patogenicidade podem ter apresentado um Rn mais alto devido ao maior número de bactérias presentes nos pulmões e, consequentemente, maior replicação no local (MEYNS et al., 2004), o que é decisivo na manifestação dos sinais clínicos e lesões, pois determinam a capacidade do agente de se multiplicar nos pulmões (THACKER & MINION, 2012).

2.2.3. Epidemiologia

A pneumonia causada por *M. hyopneumoniae* está presente em todos os países onde a suinocultura é importante (DESROSIERS, 2001). No Brasil, estima-se que 95% das granjas comerciais sejam positivas para *M. hyopneumoniae*. As exceções tendem a ser as granjas núcleo, onde seria desejável a eliminação do agente. Com relação aos planteis positivos, prevalências elevadas de lesões pulmonares em suínos de abate no Brasil revelam uma possível deficiência nas condições de manejo e instalações. Superlotação das baias, presença de excesso de poeira e gases tóxicos, temperatura e umidade inadequadas, presença de micotoxinas imunodepressoras na ração e alta pressão de infecção (por má limpeza rotineira das baias e/ou ausência ou curto vazio sanitário entre lotes) são fatores de risco importantes para a ocorrência de pneumonias. Ainda, a mistura de leitões de diferentes origens nos povoamentos de creches e terminações favorece a disseminação de diferentes cepas de *M. hyopneumoniae* pela exposição de suínos não imunes frente ao agente, favorecendo a ocorrência da doença (BARCELLOS, 2017).

A transmissão de *M. hyopneumoniae* pode ocorrer por:

- *Transmissão indireta por fômites*: forma de transmissão pouco efetiva, mas que pode ocorrer através de superfícies (BROWNE et al., 2017), ferramentas, roupas e veículos contaminados (MAES et al., 2011; DOS SANTOS et al., 2012; LOWE, 2012; THACKER & MINION, 2012);

- *Transmissão pelo ar*: Otake et al. (2010) detectaram cepas de *M. hyopneumoniae* viáveis com capacidade infectante por até 9,2 km de distância em regiões de baixo relevo, constituindo uma forma importante de transmissão do agente, visto que granjas são frequentemente encontradas próximas umas das outras;

- *Transmissão por contato direto:* através de secreções nasais e aerossois eliminados nos episódios de tosse e espirros (CALSAMIGLIA et al., 1999b). Nesse caso, a transmissão pode ocorrer horizontalmente, de leitões

infectados para suscetíveis, e das porcas para suas leitegadas (MAES et al., 2008). Dentro de um mesmo lote de animais, vários graus de excreção de *M. hyopneumoniae* podem ser encontrados. Acredita-se que suínos com alta excreção sejam mais propensos a infectar os demais através de contato direto ou por eliminação de partículas contaminadas através da tosse, espirro e respiração, contribuindo para a persistência da infecção (FLABET et al., 2010);

- *Transmissão horizontal*: essa forma de transmissão pode ocorrer entre animais infectados e suscetíveis de diferentes idades em granjas de ciclo completo; ou entre suínos de uma mesma baia na creche/terminação (CLARK et al., 1991). Uma importante forma de transmissão horizontal ocorre na creche pelo contato direto de leitões suscetíveis (não infectados) com aqueles que foram desmamados infectados por *M. hyopneumoniae*. Na ocorrência de alta prevalência de leitões positivos ao desmame, ocorre uma rápida transmissão, aumento na quantidade de animais afetados ao longo do tempo e o desenvolvimento de pneumonia precocemente, o que é observado mais frequentemente em granjas de ciclo completo. Já em sistemas de múltiplos sítios (dois sítios, três sítios ou *wean-to-finish*), a doença tende a ocorrer mais tardiamente (CALSAMIGLIA & PIJOAN, 2000; SIBILA et al., 2004; MAES et al., 2008; GIACOMINI et al., 2016). Apresentações tardias da infecção com ou sem patógenos secundários têm sido associadas à alta morbidade, mortalidade moderada, perdas econômicas significativas (SIBILA et al., 2004) e maior difusão do agente, sugerindo que o contato de suínos infectados e não infectados de diferentes idades pode favorecer a transmissão de *M. hyopneumoniae* (GIACOMINI et al., 2016). A maior severidade da doença na terminação pode estar associada a uma resposta mais aguda à infecção nessa faixa etária. Além disso, as condições encontradas principalmente na fase final de terminação aumentam a suscetibilidade do animal à infecção por *M. hyopneumoniae*, como superlotação, excesso de pó e gases, presença de micotoxinas na ração, brigas entre animais, queda de imunidade e aumento do estresse (BARCELLOS, 2017).

- *Transmissão da porca para os leitões na maternidade:* essa é uma via importante de infecção durante o período de lactação (CLARK et al., 1991;

LOWE, 2012). Embora seja possível identificar porcas mais velhas com infecção persistente por *M. hyopneumoniae* (CALSAMIGLIA & PIJOAN, 2000), a maioria desses animais já está recuperada da infecção, raramente eliminando *M. hyopneumoniae* (GOODWIN, 1965). Por outro lado, leitoas ou porcas de ordem de parto menor representam as categorias de matrizes mais importantes na transmissão de *M. hyopneumoniae*, pois são aquelas que excretam o agente com maior frequência (CALSAMIGLIA & PIJOAN, 2000; FANO et al., 2006b) e possuem menores títulos de anticorpos (MORRIS et al., 1994). Pijoan (2003) observou que 92% das porcas de ordem de parto 0 a 2 excretavam *M. hyopneumoniae* no período lactacional, enquanto que fêmeas de ordem de parto maior ou igual a 3 apresentavam apenas 20% de positividade.

Embora somente uma pequena quantidade de leitões (1,5%) possa ser colonizada na primeira semana de vida (SIBILA et al., 2007), até o desmame um número expressivo de leitões pode se infectar por aerossois ou por contato direto com a porca infectada (MAES et al., 2011; LOWE, 2012). Fano et al. (2007) observaram prevalências entre 0 e 51,28% de leitões desmamados positivos para *M. hyopneumoniae*. Além disso, Pieters et al. (2014) observaram que leitegadas cujas mães estavam excretando *M. hyopneumoniae* durante a lactação apresentavam mais chances de estarem colonizadas ao desmame e, quanto maior o período de lactação, maior a probabilidade de transmissão.

2.2.4. Sinais clínicos e lesões

A morbidade da pneumonia causada pelo *M. hyopneumoniae* é alta (40 a 60%), porém a mortalidade é baixa, no máximo 5% (NEUMANN et al., 2009) e estima-se que 80% dos suínos criados em todo o mundo estejam infectados por *M. hyopneumoniae* (FANO et al., 2005). Além disso, o período de incubação possui média de 5 semanas, e pode variar conforme o ambiente, dose infectante, suscetibilidade do animal, patogenicidade da cepa e presença de coinfecções (SCHWARTZ, 2016).

O principal sinal clínico observado é tosse seca crônica e não produtiva, normalmente induzida por exercício, com duração de 2 a 3 semanas, podendo persistir por meses no plantel (NEUMANN et al., 2009; DOS SANTOS et al., 2012; THACKER & MINION, 2012; SCHWARTZ, 2016). Como os animais permanecem por muito tempo doentes, seu desempenho pode ser prejudicado, com piora na conversão alimentar e diminuição de ganho de peso diário, que pode ser observado através da variabilidade de peso nos lotes (DOS SANTOS et al., 2012; THACKER & MINION, 2012). A presença de poeira, gases irritantes, pressão de infecção, coinfecções ou a patogenicidade da cepa envolvida podem agravar e prolongar o quadro clínico (NEUMANN et al., 2009; SIBILA et al., 2009; THACKER & MINION, 2012), sendo possível observar dispneia, tosse produtiva e maiores taxas de mortalidade (DOS SANTOS et al., 2012). Devido ao curso longo da doença (SIBILA et al., 2009; THACKER & MINION, 2012) e à presença de imunidade materna, os sinais clínicos dificilmente são observados antes das seis semanas de idade, ocorrendo com maior frequência nas fases de crescimento e terminação (SIBILA et al., 2009). No entanto, na ocorrência de surtos em granjas livres, animais adultos e leitões jovens também podem apresentar sinais clínicos, que geralmente são mais graves, como pirexia, anorexia, dispneia, tosse seca e aumento de mortalidade (DOS SANTOS et al., 2012).

A pneumonia causada pelo *M. hyopneumoniae* se caracteriza por uma broncopneumonia supurativa ou catarral (LÓPEZ, 2007), em que são observadas áreas rígidas e escuras (avermelhadas, quando aguda; ou cinzentas e/ou fibrosas, quando crônica) denominadas áreas de consolidação pulmonar, que cicatrizam em 10-12 semanas quando não complicadas por outros patógenos (SCHWARTZ, 2016). Geralmente as lesões são localizadas, características de infecções aerógenas, e têm distribuição crânio ventral, afetando os lobos apicais, cardíacos, intermediário e a porção cranial dos diafragmáticos. Esse padrão de distribuição ocorre por influência gravitacional e pela menor eficiência dos mecanismos de defesa nas porções craniais do pulmão (LÓPEZ, 2007). A monitoria de abate pode ser uma prática utilizada pelo médico veterinário para auxiliar no diagnóstico de pneumonias e para determinar a prevalência e intensidade de lesões pulmonares ao abate.

Geralmente são observadas lesões com diversos graus de intensidade, o que pode estar relacionado à grande variação no número de animais infectados ao desmame (RUIZ et al., 2003) e à difusão lenta do *M. hyopneumoniae* (FANO et al., 2007). Michiels et al. (2017) observaram que a intensidade e frequência de lesões pulmonares foi proporcional ao número de variantes de *M. hyopneumoniae* encontradas nos lotes de suínos abatidos, sugerindo que lotes de animais com menor variedade de cepas tendem a apresentar melhor saúde pulmonar.

Ao exame histopatológico observa-se infiltrado inflamatório mononuclear nas paredes alveolares, além de macrófagos e neutrófilos no lúmen de brônquios e bronquíolos (LÓPEZ, 2007, REDONDO et al., 2009). Perda de cílios em células epiteliais e proliferação do epitélio de brônquios e bronquíolos também podem ser encontradas (THACKER & MINION, 2012). A hiperplasia de BALT é comumente observada e dependendo da intensidade desta lesão, pode haver compressão e até mesmo oclusão de brônquios e bronquíolos (LÓPEZ, 2007; NEUMANN et al., 2009; REDONDO et al., 2009; TAKEUTI et al., 2013).

2.2.5. Diagnóstico

O histórico, sinais clínicos e lesões macroscópicas têm sido usados como critérios de diagnóstico da infecção por *M. hyopneumoniae*. No entanto, o diagnóstico laboratorial é fundamental (NEUMANN et al., 2009). A seguir serão descritas algumas técnicas utilizadas no diagnóstico de *M. hyopneumoniae*.

2.2.5.1. Exame bacteriológico

O isolamento de *M. hyopneumoniae* é considerado “padrão ouro” no diagnóstico de *M. hyopneumoniae* (SIBILA et al., 2009), porém não é uma técnica empregada rotineiramente, devido à dificuldade em realizá-la (MAES et al., 2008). Micoplasmas são micro-organismos fastidiosos e requerem condições específicas para seu crescimento (MARKEY et al., 2013), que pode

ser diretamente influenciado pelo meio utilizado, pela quantidade de *M. hyopneumoniae* e pela presença de outras bactérias nas amostras (ANDERSON et al., 2016). Dessa forma, se faz necessária a utilização de antimicrobianos, como a penicilina, aos meios de cultivo para inibir bactérias Gram-positivas (MARKEY et al., 2013). Além disso, após o isolamento de colônias em meio de cultura, a confirmação deve ser realizada pela Reação em Cadeia de Polimerase (PCR), já que outras espécies de micoplasmas crescem mais rapidamente e podem interferir no diagnóstico. É comum o isolamento de *M. hyorhinis* a partir de lesões pulmonares semelhantes àquelas causadas por *M. hyopneumoniae* (FRIIS, 1975; THACKER & MINION, 2012). Apesar das dificuldades no isolamento de *M. hyopneumoniae*, Anderson et al. (2016) obtiveram uma taxa de sucesso de isolamento de 25 a 100%. Basicamente, a técnica se caracterizou pela identificação primária de animais com sinais clínicos sugestivos de infecção por *M. hyopneumoniae*, coleta de suabe de laringe para realização de PCR Real Time, eutanásia de animais que apresentaram resultados de Ct médio entre 28.48 a 35.91 ao PCR, e coleta de pulmões com lesões sugestivas de pneumonia micoplásmica. No laboratório, os pulmões eram congelados e descongelados para processamento e cultivo em meio de cultura Friis modificado (ANDERSON et al., 2016).

2.2.5.2. Diagnóstico molecular

A dificuldade de isolamento de *M. hyopneumoniae* levou ao desenvolvimento de técnicas moleculares de diagnóstico, como a PCR (SIBILA et al., 2009; MAROIS et al., 2010). O monitoramento de *M. hyopneumoniae* em suínos vivos através de PCR constitui uma ferramenta importante para fornecer informações a respeito da dinâmica de infecção nos planteis e do momento em que os animais se infectam (CALSAMIGLIA et al., 1999a; FLABET et al., 2010). Além disso, possibilita a detecção de animais positivos antes mesmo da soroconversão (CALSAMIGLIA et al., 1999a; DEEGAN et al., 2016) e da manifestação dos sinais clínicos (PIETERS & PIJOAN, 2006). Após inoculações experimentais, observou-se que a detecção de *M. hyopneumoniae*

por PCR pode começar 11 dias após a infecção, enquanto que a detecção de anticorpos se iniciou somente após 34 dias (PIETERS et al., 2009).

Diversas técnicas foram desenvolvidas, no entanto nested-PCR e PCR Real Time são as técnicas mais utilizadas tanto no diagnóstico de animais infectados, quanto no estudo da dinâmica de infecção nos planteis. Nested-PCR é uma técnica qualitativa e de alta especificidade (CALSAMIGLIA et al., 1999b) e PCR Real Time possui alta sensibilidade, é um teste rápido e apresenta menor chance de contaminação (MAROIS et al., 2010). Além disso, é um teste quantitativo, indicando o número de ciclos necessários (Ct - *Cycle Threshold*) para se detectar a sequência alvo (SCHWARTZ, 2016).

A detecção de *M. hyopneumoniae* aumenta com a profundidade do trato respiratório. Quanto mais inferior a coleta, mais sensível será a amostra, já que a bactéria se liga ao epitélio ciliado, se multiplica na traqueia, brônquios e bronquíolos (BLANCHARD et al., 1992) e quanto mais células ciliadas em contato com a amostra, maior a sensibilidade (FLABET et al., 2010). Marois et al. (2010) observaram que a quantidade de *M. hyopneumoniae* na traqueia, similar àquela encontrada nos pulmões, é de 10 a 10 mil vezes maior do que na cavidade nasal, evidenciando a importância da escolha do local para coleta de amostras no diagnóstico dessa infecção. O suabe nasal foi utilizado por muitos anos (CALSAMIGLIA et al., 1999b; CALSAMIGLIA & PIJOAN, 2000; RUIZ et al., 2003; SIBILA et al., 2004; FANO et al., 2007) no diagnóstico de infecções por *M. hyopneumoniae* pela praticidade na coleta do material. No entanto, foi observado que a cavidade nasal não é o local de predileção do agente (PIETERS & PIJOAN, 2006) e que a excreção nasal é intermitente, sendo possível detectar *M. hyopneumoniae* somente por um período limitado de tempo (CALSAMIGLIA et al., 1999b; PIETERS & PIJOAN, 2006). Dessa forma, esse local deve ser evitado para fins de diagnóstico, já que é possível que resultados falsos negativos ocorram (MEYNS et al., 2004). Pieters et al. (2017) compararam quatro locais (suabe nasal, suabe laríngeo, lavado traqueobrônquico e fluído oral) para obtenção de amostras para diagnóstico da infecção experimental por *M. hyopneumoniae* através de PCR Real Time e observaram que os suabes de laringe foram mais sensíveis em detectar

infecções iniciais de *M. hyopneumoniae* quando comparados aos outros tipos de amostras. O segundo melhor tipo de amostra foi lavado traqueobrônquico, seguido do suabe nasal e fluído oral. Em outro estudo, Sievers et al. (2015) observaram maior sensibilidade em suabes bronquiais, seguidos dos suabes laríngeos. A vantagem da utilização do suabe laríngeo é a coleta *in vivo*, permitindo que estudos de dinâmica de infecção de *M. hyopneumoniae* sejam realizados longitudinalmente. Em condições experimentais, *M. hyopneumoniae* pode ser detectado em 81% e 100% das amostras obtidas através de suabe de laringe 5 e 28 dias após infecção, respectivamente (PIETERS et al., 2017). Já em infecções naturais por contato direto, a detecção de *M. hyopneumoniae* pode ser obtida em 41% dos animais após 14 dias de exposição (ROOS et al., 2016).

2.2.5.2. Sorologia

Os métodos sorológicos de diagnóstico de *M. hyopneumoniae* são normalmente realizados através de ensaio imunoenzimático (ELISA), utilizado com frequência no monitoramento de planteis, principalmente em granjas negativas (SIBILA et al., 2009; MORES, 2010). O ELISA é considerado o melhor teste sorológico, pois detecta todas as classes de imunoglobulinas, é rápido, permite testar um grande número de animais vivos, é quantitativo, e apresenta alta especificidade, embora reações cruzadas com *M. flocculare* possam ocorrer (CALSAMIGLIA et al., 1999a). O teste apresenta algumas limitações, como: impossibilidade de detecção de infecções iniciais; não diferencia anticorpos maternos, vacinais ou produzidos após exposição natural (SIBILA et al., 2009), e embora possibilite a detecção de anticorpos, não esclarece o momento da infecção (CALSAMIGLIA et al., 1999a; PIJOAN, 2003). Uma estimativa pode ser feita, já que os anticorpos começam a diminuir abaixo de limites detectáveis cerca de três meses após a vacinação (MAES et al., 1999).

Como *M. hyopneumoniae* adere aos cílios do epitélio respiratório e não invade o parênquima pulmonar, a apresentação do antígeno ao hospedeiro é

lenta, levando a um atraso na soroconversão (SIBILA et al., 2009). O tempo para os animais soroconverterem depende da cepa envolvida ou do tipo de infecção (SORENSEN et al., 1997). Animais infectados com cepas mais virulentas apresentam soroconversão mais rápida, possivelmente devido a uma maior imunogenicidade ou multiplicação mais rápida (MEYNS et al., 2004; VICCA et al., 2003). Em inoculações experimentais, Sorensen et al. (1997) e Pieters et al. (2017) observaram soroconversão aos 28 e 21 dias pós inoculação, respectivamente. Já Deegan et al. (2016) observaram que após 75 a 100 dias do início do contato natural direto de animais livres com infectados, 100% soroconverteram. O tempo de soroconversão pode variar de acordo com a dose infectante, que é maior em condições experimentais, e com o tipo de transmissão. Animais infectados após contato direto apresentam soroconversão mais rápida (após 28 dias) em relação àqueles que tiveram contato indireto (após 42 dias) com animais positivos, devido à colonização mais lenta e doses infectantes mais baixas (FANO et al., 2005).

2.2.6. Controle

Eliminar *M. hyopneumoniae* dos planteis reflete em altos custos e em práticas de biossegurança rígidas para a manutenção das granjas livres. Dessa forma, geralmente é necessário conviver com a doença e controlá-la para que os prejuízos econômicos ocorram em níveis baixos (DOS SANTOS et al., 2012). Como *M. hyopneumoniae* geralmente está associado a outros agentes, as medidas de controle são diversas. As principais incluem: uso de antimicrobianos de forma preventiva e curativa, vacinação e correção de fatores de risco (MAES et al., 2008; MORES, 2010), que serão abordadas a seguir.

2.2.6.1. Terapia antimicrobiana

Os micoplasmas não possuem parede celular, dessa forma, classes de antimicrobianos que têm ação sobre essa estrutura, como os β-lactâmicos (penicilinas e cefalosporinas), não podem ser utilizados no tratamento e controle da infecção por *M. hyopneumoniae*. Os grupos dos macrolídeos, lincosamidas, anfenicois, fluoroquinolonas, pleuromutilinas e tetraciclinas são os mais utilizados (MAES et al., 2008). As fluoroquinolonas; combinações de tetraciclina com tiamulina ou com lincomicina (DESROSIERS, 2001); tiamulina; tilosina, e valnemulina têm demonstrado boa eficácia no tratamento e controle dessa bactéria (BURCH, 2004). Por se tratar de uma infecção associada frequentemente a outros patógenos, incluindo bactérias Gram-negativas, antimicrobianos ativos para Gram-negativas ou associações antimicrobianas de amplo espectro devem ser utilizadas (MAES et al., 2008). Drogas da classe dos macrolídeos com espectro estendido de ação (Gamitromicina, Tilmicosina, Tulatromicina e Tildipirosina) também têm apresentado resultado satisfatório no controle da infecção pelo *M. hyopneumoniae* (BARCELLOS, 2017).

A terapia antimicrobiana pode ser realizada de forma preventiva, através de pulsos na ração ou na água nas fases mais críticas da produção (creche e terminação); ou injetável, no tratamento de animais enfermos ou de todos os animais nas baias afetadas (DOS SANTOS et al., 2012). Embora não impeça a infecção e aderência do *M. hyopneumoniae* nos cílios do trato respiratório (LE GRAND & KOBISCH, 1996), essa prática pode ser utilizada em planteis com alto desafio, gerando, geralmente, bons resultados (MATEUSEN et al., 2002). Suínos desafiados com *M. hyopneumoniae* e posteriormente tratados com tilosina via ração por 21 dias apresentaram sinais clínicos e lesões pulmonares mais brandas quando comparados a animais desafiados e não tratados (VICCA et al., 2005). Além disso, o uso de medicação estratégica em matrizes pode ser uma forma eficaz de controle para diminuir a excreção desses animais durante o período de lactação (MAES et al., 2008). Painter et al. (2012) observaram que a associação entre vacinação pré-desmame para *M. hyopneumoniae* e o uso de tulatromicina na terminação não resultou na eliminação de animais positivos, porém uma menor frequência de suínos positivos para *M.*

hyopneumoniae em idade de terminação foi observada quando comparada aos animais que foram apenas vacinados. Outro estudo, conduzido por Holst et al. (2013), concluiu que a aplicação de tulatromicina em matrizes pré-parto reduziu a excreção de *M. hyopneumoniae* por porcas ao parto e ao desmame e resultou em um menor número de leitegadas desmamadas positivas, provavelmente pela diminuição na quantidade de bactérias no trato respiratório.

As desvantagens da terapia antimicrobiana incluem: o possível reaparecimento dos sinais clínicos quando o tratamento cessa, e a possibilidade de aumento de resistência antimicrobiana quando utilizada por longos períodos (MAES et al., 2011). Devido à dificuldade de isolamento da bactéria, poucos trabalhos sobre suscetibilidade antimicrobiana de *M. hyopneumoniae* são encontrados na literatura. Esses estudos revelaram resistência dos isolados de *M. hyopneumoniae* a diversas classes de antimicrobianos, como às tetraciclinas (BURCH, 2004), aos macrolídeos, lincosamidas e fluoroquinolonas (VICCA et al., 2004).

2.2.6.2. Vacinação

A vacinação com bacterinas é amplamente utilizada em sistemas tecnificados no controle da infecção por *M. hyopneumoniae* (MAES et al., 2008). Embora não se saiba exatamente quais os mecanismos envolvidos na proteção gerada pelas vacinas, acredita-se que ocorra tanto a nível humoral, através do aumento da produção de anticorpos específicos (IgG e IgA) e redução da produção de citocinas pró-inflamatórias, quanto a nível celular, através da produção de células secretoras de IFN-ɣ no sangue (PARK et al., 2016a), responsáveis pela ativação de macrófagos (THACKER et al., 2000). Ainda, acredita-se que a imunidade celular corresponda ao principal mecanismo de proteção frente ao *M. hyopneumoniae* (THACKER et al., 2000; PARK et al., 2016a).

O uso de vacinas, associado a boas práticas de manejo, pode gerar bons resultados, já que a doença é multifatorial (HAESEBROUCK et al., 2004; MAES et al., 2011). Embora as vacinas não impeçam a colonização

(THACKER et al., 1998), nem diminuam a transmissão de *M. hyopneumoniae* entre suínos infectados e suscetíveis (MEYNS et al., 2006), os benefícios são diversos e incluem a diminuição da carga bacteriana nos pulmões (WOOLEY et al., 2014), aumento de 2 a 8% no ganho de peso diário, melhora na conversão alimentar (2 a 5%), redução de mortalidade de animais (MAES et al., 1998), diminuição na excreção do agente, sinais clínicos, intensidade e prevalência de lesões pulmonares (PARK et al., 2016a). No entanto, esses ganhos são geralmente atingidos somente após diversos meses após o início da vacinação (HAESEBROUCK et al., 2004).

Normalmente a vacinação é feita em leitões, animais de reposição e, ocasionalmente, em matrizes em final de gestação (CALSAMIGLIA & PIJOAN, 2000; FANO et al., 2006b).

Vacinação de leitões: é utilizada frequentemente no controle da infecção por *M. hyopneumoniae*. Inicialmente acreditou-se que a vacinação para leitões com duas doses fosse mais eficaz, porém foi observado que os protocolos com uma ou duas doses podem conferir proteção similar (BACCARO et al., 2006). Como a vacinação com uma dose é menos laboriosa, tem sido utilizada com frequência (MAES et al., 2008). Ainda, a aplicação de vacinas bivalentes para *M. hyopneumoniae* e PCV2 tem gerado boa proteção frente aos dois patógenos (PARK et al., 2016a).

O tipo de sistema de produção, o manejo adotado e a dinâmica de infecção são determinantes no estabelecimento do melhor protocolo de vacinação para *M. hyopneumoniae* para cada granja (HAESEBROUCK et al., 2004; MAES et al., 2008; PIETERS & SIBILA, 2017). Em sistemas de criação de ciclo completo, a infecção tende a ser mais precoce (maternidade), pela transmissão progressiva e constante entre os animais suscetíveis e infectados (SIBILA et al., 2004; GIACOMINI et al., 2016). Dessa forma, a vacinação dos leitões deve ser feita antes de 4 semanas de idade e a vacinação de porcas pré-parto poderia gerar bons resultados. Já em sistemas de múltiplos sítios, preconiza-se a vacinação entre 4 e 10 semanas de idade, pois as infecções tendem a ser mais tardias devido à interrupção na transmissão horizontal de *M. hyopneumoniae*, já que os animais são separados e reagrupados

(HAESEBROUCK et al., 2004; SIBILA et al., 2004; MAES et al., 2008). No entanto, geralmente os leitões são vacinados ao desmame pela facilidade de manejo e pela necessidade da aplicação concomitante de vacinas que conferem proteção a outros patógenos (PIETERS & SIBILA, 2017). Como o desmame é um período estressante na vida dos leitões, Arsenakis et al. (2017) avaliaram se a vacinação para *M. hyopneumoniae* três dias antes do desmame resultaria em melhores resultados quando comparada ao protocolo convencional (vacinação ao desmame). Foi observado um maior ganho de peso diário durante toda a terminação e das duas às 27 semanas de idade de vida dos leitões vacinados três dias pré-desmame. No entanto, não foram observadas diferenças significativas na prevalência e severidade de lesões pneumônicas ao abate entre os diferentes protocolos vacinais testados, o que pode estar relacionado a coinfecções com outros patógenos respiratórios.

Vacinação de animais de reposição: a vacinação de leitoas de reposição é recomendada para animais livres de *M. hyopneumoniae* ou que sejam provenientes de granjas com baixo desafio com o objetivo principal de evitar a desestabilização do plantel que irá receber os animais (MAES et al., 2011). Michiels et al. (2017) observaram uma maior prevalência de lesões pulmonares em suínos provenientes de granjas em que a vacinação de leitoas de reposição não era realizada, tornando-se um fator de risco na ocorrência de pneumonias em granjas. Normalmente, leitoas são vacinadas por volta dos 150 dias de idade, quando são geralmente introduzidas nos planteis.

Vacinação de matrizes: Ruiz et al. (2003) observaram que matrizes vacinadas 5 e 3 semanas pré-parto foram capazes de transferir imunidade passiva às suas leitegadas via colostro e uma menor prevalência de leitões desmamados positivos para *M. hyopneumoniae* por PCR foi observada, o que pode estar relacionado a uma menor excreção da bactéria pelas porcas vacinadas ou à imunidade passiva transferida aos leitões cujas mães foram vacinadas. Sibila et al. (2006) também utilizaram o mesmo protocolo vacinal em matrizes pré-parto e observaram uma redução de lesões pulmonares ao abate em leitões cujas mães foram vacinadas quando comparados a leitões de matrizes não vacinadas.

2.2.6.3. Correção de fatores de risco

A correção dos fatores de risco também é fundamental no controle da infecção por *M. hyopneumoniae* (DESROSIERS, 2001) e deveria ser a primeira medida adotada (MAES et al., 2011), pois possibilita a diminuição da transmissão do agente ou reduz os danos pulmonares por agentes secundários (MAES et al., 2008). A seguir serão abordadas práticas de manejo que podem ser utilizadas no controle da infecção por *M. hyopneumoniae*:

- A introdução de animais de reposição é uma prática comum na suinocultura e pode acarretar problemas sanitários importantes, tanto para os suínos do plantel, quanto aos que estão sendo introduzidos, principalmente quando ocorre a entrada de animais negativos em planteis positivos para *M. hyopneumoniae* (PIETERS & FANO, 2016). O aumento na taxa de reposição de leitoas, mesmo que vacinadas, aumenta a ocorrência de doença clínica, desestabilizando o plantel (FANO et al., 2006a) e aumentando a variabilidade de cepas circulantes nas granjas (VRANCKX et al., 2011). Como a renovação do plantel é necessária, a introdução de leitoas deveria ser realizada a partir de granjas com *status* sanitário semelhante, e a quarentena e adaptação deveriam ser empregadas (AMASS & BAYSINGER, 2006). A aclimatação é uma medida importante que pode auxiliar no controle da infecção (PIETERS & FANO, 2016), especialmente para leitoas de reposição livres de *M. hyopneumoniae* introduzidas em planteis positivos. Considerando-se que a infecção por *M. hyopneumoniae* é lenta, que os animais ficam infectados por um longo período (PIETERS et al., 2009), e que a taxa de transmissão é baixa (MEYNS et al., 2004), a exposição de leitoas negativas, deveria ser iniciada por volta dos 50 dias de idade através do contato direto de animais livres com excretores de *M. hyopneumoniae* (PIETERS & FANO, 2016). Como o início da infecção ocorre entre 4 e 6 semanas após a exposição e a excreção pode durar aproximadamente 240 dias (PIETERS et al., 2009), haveria tempo para que os animais se recuperassem e cessassem a excreção de *M. hyopneumoniae* até a data do primeiro parto, diminuindo assim a transmissão que ocorre na maternidade (PIETERS & FANO, 2016). Ainda, Roos et al. (2016) sugerem que o melhor cenário para expor naturalmente leitoas livres de *M. hyopneumoniae*

em uma baia com 10 animais seria o contato de 6 leitoas que estivessem eliminando o agente com 4 negativas em um período de 4 semanas. Embora seja eficaz, esse método é inicialmente laborioso, pois implica na identificação prévia de animais excretores. Além disso, esse cenário de infecção se aplica a uma variante específica utilizada no experimento. Um grande número de variantes de *M. hyopneumoniae* circulando em determinados rebanhos já foi descrita (DOS SANTOS et al., 2015) e a dinâmica de infecção pode variar de acordo com sua patogenicidade (VICCA et al., 2003);

- O manejo “todos dentro, todos fora” é uma das medidas de controle mais importantes, pois permite a realização de limpeza e desinfecção das instalações entre lotes, diminuindo a pressão de infecção e interrompendo a transmissão do agente entre animais de diferentes idades (CLARK et al., 1991);

- A alta densidade animal favorece o estresse e a transmissão de doenças infecciosas, principalmente respiratórias. Na terminação, a densidade recomendada em instalações com piso totalmente ripado deve ser igual ou superior a 0,7m^2/animal (MAES et al., 2008). No Brasil, normalmente se utiliza densidade entre 0,9 a 1m^2/animal em baias com piso semi-ripado ou com lâmina d’água (BARCELLOS et al., 2017);

- A mistura de animais e o maior número de origens implicam também no contato de animais suscetíveis com infectados (MAES et al., 2008), além do contato com novas cepas de *M. hyopneumoniae* ou agentes aos quais os animais não possuíam imunidade;

- A manutenção de temperatura, umidade (60-80%) e ventilação adequadas nas instalações é outra medida fundamental no controle de qualquer doença respiratória (BARCELLOS et al., 2008; DOS SANTOS et al., 2012). Em um estudo realizado por Browne et al. (2017), foi demonstrada a viabilidade de seis isolados *M. hyopneumoniae* por até oito dias a 4ºC em diferentes tipos de superfície, enquanto que a sobrevivência da bactéria foi observada por no máximo dois dias quando incubada a temperaturas de 25ºC ou 37ºC, demonstrando que *M. hyopneumoniae* possui resistência a baixas

temperaturas fora do hospedeiro, contribuindo na transmissão indireta do agente. Essa característica pode estar relacionada com a ausência de parede celular dos micoplasmas, tornando-os suscetíveis a altas temperaturas (FRIIS, 1974). Além disso, a viabilidade do micro-organismo foi favorecida na presença de poeira, que pode atuar como um vetor na transmissão de *M. hyopneumoniae* (BROWNE et al., 2017);

- O controle de outras doenças respiratórias ou imunossupressoras (como PCV2 e IAV) também pode ser empregado para evitar a queda de imunidade dos animais e a consequente infecção por *M. hyopneumoniae*, ou ainda de patógenos que potencializem o quadro clínico, como *P. multocida* e *H. parasuis* (MORES, 2010);

- Medidas de biossegurança, como: controle de moscas e roedores, e restrição na entrada de pessoas, veículos e equipamentos devem ser empregadas, já que a transmissão indireta pode ocorrer (MAES et al., 2008);

- O desmame dos leitões entre 7 e 10 dias reduz, porém não elimina, a transmissão da porca para sua leitegada (DRITZ et al., 1996; MAES et al., 2008). No entanto, não é empregado a campo, já que não é permitido em alguns países e pode prejudicar a qualidade do leitão desmamado (MAES et al., 2008);

- O momento de realização da uniformização de leitegada deve ser respeitado, pois a imunidade celular da porca, importante na proteção frente ao *M. hyopneumoniae*, é absorvida somente por seus próprios leitões (BANDRICK et al., 2011).

2.2.6.4. Erradicação

Na Europa (Dinamarca, Suíça, Suécia e Finlândia) o método mais utilizado na erradicação de *M. hyopneumoniae* é conhecido como “Método Suíço de Depopulação” e vem apresentando bons resultados (MAES et al., 2008; NEUMANN et al., 2009; THACKER & MINION, 2012). O protocolo

consiste na eliminação de animais com menos de 10 meses de idade, seguida da interrupção dos partos por pelo menos duas semanas e medicação dos animais remanescentes no plantel (HOLST et al., 2015) com produtos como lincomicina, tiamulina, tulatromicina ou a combinação de clortetraciclina, tilosina e sulfas (MAES et al., 2008; NEUMANN et al., 2009; THACKER & MINION, 2012). No entanto, o alto custo, as reinfecções frequentes (2,6 a 10% ao ano; THACKER & MINION, 2012), além da interrupção na produção de leitões, dificultam a execução dessa prática (MAES et al., 2008). Outro método de eliminação de *M. hyopneumoniae* consiste na exposição de todo o plantel reprodutivo frente à bactéria, seguida do fechamento da granja por 8 meses, na vacinação maciça do plantel para *M. hyopneumoniae*, e na medicação dos animais com antimicrobianos (tulatromicina) antes da chegada de leitoas livres (HOLST et al., 2015). Yeske (2014) comparou esses dois métodos de erradicação e observou que a depopulação associada à medicação foi mais eficiente (89% de sucesso) e possibilitou que mais granjas permanecessem negativas por um período mais longo do que somente a utilização de medicação (67% de sucesso). Outros métodos de erradicação incluem a depopulação total e repopulação com animais livres de *M. hyopneumoniae*, permitindo a erradicação de outros patógenos concomitantemente (HOLST et al., 2015). Ainda, a medicação maciça dos animais sem a necessidade do fechamento da granja pode ser empregada. Neste caso, todo o plantel é medicado com um antimicrobiano de longa ação com reforço após duas semanas. Na ocorrência de novos partos durante o período de eliminação, leitões também são medicados ao parto e 14 dias após o nascimento (HOLST et al., 2015).

3. CAPÍTULO II – PRIMEIRO ARTIGO CIENTÍFICO

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Detection of *Mycoplasma hyopneumoniae* in naturally infected gilts over time

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ABSTRACT

Mycoplasma hyopneumoniae causes a chronic respiratory infection in pigs and its transmission occurs mainly by direct contact and by vertical transmission (sow-to-piglet). The objective of this study was to assess the detection dynamics and persistence of *M. hyopneumoniae* natural infection in replacement gilts. Forty-four twenty-day-old gilts were selected from a *M. hyopneumoniae* positive farm and followed up to one day prior to their first weaning. Laryngeal swabs were collected every 30 days, starting at day 20, for *M. hyopneumoniae* detection by real-time PCR, resulting in 12 samplings. Piglets born to selected females were sampled via laryngeal swabs one day prior to weaning to evaluate sow-to-piglet transmission. The *M. hyopneumoniae* prevalence was estimated at each one of the 12 samplings in gilts and a multiple comparison test and Bonferroni correction were performed. Bacterial detection in gilts started at 110 days of age (doa) and a significant increase ($p < 0.05$) occurred at 140 doa. The *M. hyopneumoniae* prevalence remained above 20% from 140 to 230 doa, decreasing thereafter. However, it did not reach 0% at any sampling after 110 doa. In this study, *M. hyopneumoniae* was not detected in piglets sampled prior to weaning. The *M. hyopneumoniae* detection pattern showed that in natural infections, gilts were positive for *M. hyopneumoniae* for one to three months, but occasionally long-term detection may occur. Moreover, the lack of *M. hyopneumoniae* detection throughout the study in 18.2% of gilts indicated the existence of negative subpopulations in positive herds.

1. Introduction

Mycoplasma hyopneumoniae (*M. hyopneumoniae*), which causes a chronic bronchopneumonia in pigs (enzootic pneumonia; Goodwin et al., 1965; Mare and Switzer, 1965), plays an important role in the porcine respiratory disease complex (PRDC), and it is associated with large economic losses in pig production worldwide (Thacker and Minion, 2012). Infections caused by *M. hyopneumoniae* are considered among the most prevalent bacterial respiratory infections in pigs and are characterized by chronicity.

Transmission of *M. hyopneumoniae* occurs mainly by direct contact among pigs of similar ages or production stages (Clark et al., 1993; Fano et al., 2005), and by vertical transmission, from dams to piglets (Calsamiglia and Pijoan, 2000). Despite the *M. hyopneumoniae* transmission rate being low (Meyns et al., 2004; Pieters et al., 2010; Villarreal et al., 2011), it is high enough for infections to be maintained within herds. Once infected, pigs shed *M. hyopneumoniae* for extended periods of time that can reach up to 214 days, even when they become asymptomatic carriers, and are capable of infecting susceptible pigs (Pieters et al., 2009). This feature is especially important in animals that remain in the herd for long time, as it is the case of reproductive females.

Identifying the time points when animals in the reproductive herd are actually infected and shedding *M. hyopneumoniae* is animportant aspect to understand disease transmission among dams and the potential for exposure and infection in newborn pigs. For example, the presence of different sow infectious status for porcine reproductive and respiratory syndrome virus (PRRSv) in endemically infected farms, described by Dee et al. (1996) suggested the presence of negative subpopulations within the breeding herd. The existence of negative populations was thought to be one risk factor for viral transmission perpetuation. Thus, a clear definition of the critical opportunities for infection is key in order to develop and apply measures directed at disease control.

The use of PCR to detect *M. hyopneumoniae* in live pigs provides information about the infection dynamics of this bacterium, since it is possible to identify infected pigs, even before seroconversion occurs (Calsamiglia et al., 1999; Roos et al., 2016; Pieters et al., 2017) or prior

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to the observation of clinical signs (Pieters and Pijoan, 2006). Moreover, the use of laryngeal swabs (Pieters et al., 2017), tracheo-bronchial mucus samples, (Vangroenweghe et al., 2015), and tracheo-bronchial swabs (Fablet et al., 2010) has improved the sensitivity to detect *M. hyopneumoniae* by PCR in live pigs.

Longitudinal studies coupled with sensitive diagnostics are useful tools to provide information about population infection dynamics and can aid the application of disease mitigation measures, such as management practices, vaccination, medication, and elimination (Maes et al., 2008). However, the use of sensitive *in vivo* sampling techniques for detection of *M. hyopneumoniae*, such as laryngeal swabs, has only become popular and validated in recent years and to the best of our knowledge, comprehensive field investigations including sampling during the acute and chronic phases of infection are lacking in the literature. Therefore, the objective of this study was to assess the detection dynamics and persistence of *M. hyopneumoniae* natural infection in replacement gilts.

2. Materials and methods

2.1. Ethics statement

The farm system participating in this study followed its own management practices. Additionally, all sampling protocols that were part of the investigation were approved by the Institutional Animal Care and Use Committee of the Federal University of Rio Grande do Sul.

2.2. Animals and housing

Eighty twenty-day-old gilts (Landrace x Large White) were selected from a *M. hyopneumoniae* positive multiplier pig farm. Farm selection was based on respiratory clinical symptoms and laboratorial diagnostic. Briefly, discussions with the attending veterinarian suggested respiratory disease with observation of clinical signs (dry cough). In addition, diagnostic testing including serology and lung lesion evaluation were performed. The farm was located in southern Brazil, where most of the pork production of the country takes place. Gilts were followed from selection up to one day prior to their first weaning event, when they were 326–358 days of age (doa). Gilts were housed in three different sites through the study (Fig. 1): a) Multiplier herd (1500 sows): from birth to 63 doa. b) Finishing unit (1400 gilts): from 63 to 168 doa (located 65 kilometers apart from the multiplier). c) Farrowing unit (1800 sows): from 169 doa to breeding (approximately 210 doa), farrowing (approximately 324 doa) or weaning (located 500 m apart from the finishing unit). Selected gilts were weaned at 21 doa and housed in three or five pens (nursery, and finishing or farrowing unit up to first service, respectively) along with other gilts from the same batch which were not included in this study. The study took place from October 2016 until September 2016.

The vaccination protocol for *M. hyopneumoniae* consisted on two doses of a commercial bacterin (M + PAC®; MSD Saúde Animal) at 21 and 150 doa. Also, gilts were vaccinated against Porcine Circovirus type 2 (PCV-2; Circumvent PCV®, MSD Saúde Animal) at 21 doa. The farms from this study were also endemic and infected with *Pasteurella multocida*, *Actinobacillus pleuropneumoniae, Haemophilus parasuis* and Influenza A virus (IAV), and sub clinically infected with PCV-2, but negative for PRRSv. A total of 220 piglets, five piglets per litter, born to the selected dams were also enrolled in the study.

Antibiotic treatments were administered to gilts in the study as part of the farm management. Briefly, gilts were treated with antibiotics in the nursery and in the finishing phase. Antibiotics were administered in feed with drugs affecting Mycoplasma species. Piglets were not treated with antibiotics affecting Mycoplasmas at any point in time.

2.3. Experimental design

Gilts were randomly selected and ear tagged at the beginning of the study. Laryngeal swabs were collected from the gilts every 30 days, to be tested for *M. hyopneumoniae* by real-time PCR, resulting in 12 samplings for each gilt (Fig. 1). Piglets were sampled via laryngeal swabs one day prior to weaning to evaluate sow-to-piglet transmission. Cross-fostering was not allowed during the course of this study to guarantee sampling piglets on their biological dams.

2.4. Sample collection

Laryngeal swabs were obtained from replacement gilts and five of their piglets by the introduction of sterile rayon swabs (Laborclin®,

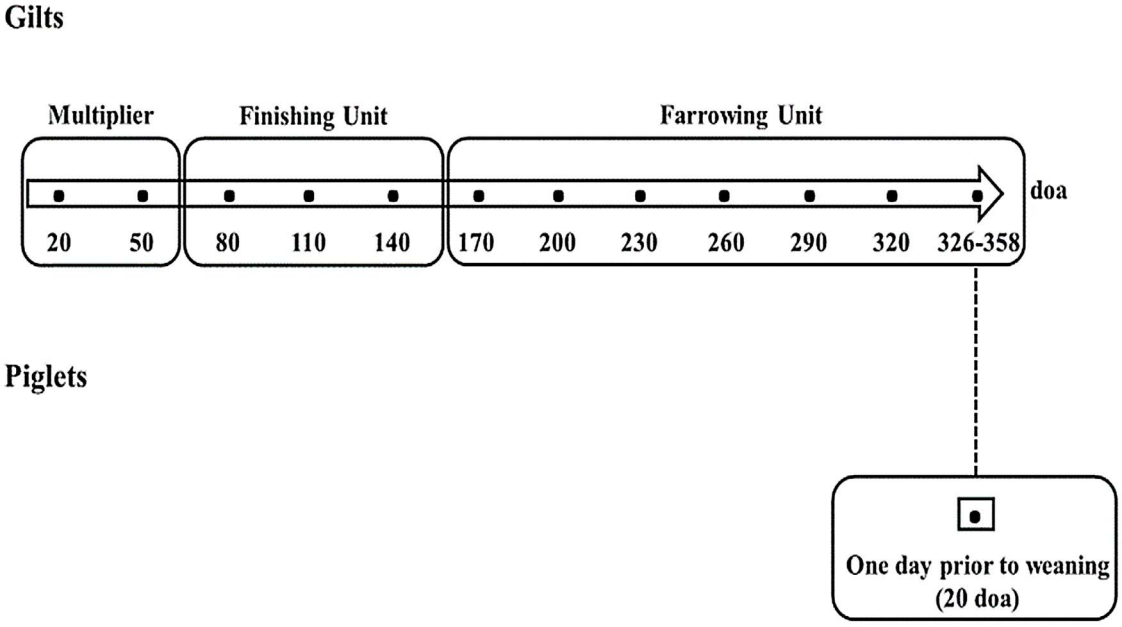

Fig. 1. Graphic representation of the experimental design and housing location of the female replacement gilts selected for the study. Number represent days of age (doa) when samples were collected. Laryngeal swabs were collected every 30 days, starting at 20 doa, up to one day prior to the weaning event and were processed for real-time PCR testing for *M. hyopneumoniae*, totaling 12 samplings (black dots). Five piglets born to each dam were sampled one day prior to weaning in order to evaluate sow-to-piglet transmission.

Pinhais, PR, Brazil) into the mouth cavity until they reached the larynx, using a mouth gag and a laryngoscope (Pieters et al., 2017). All swabs were labelled, refrigerated during transport, and stored at −20 °C in the laboratory until DNA extraction was performed.

2.5. Sample processing and testing

Real-time PCR for *M. hyopneumoniae:* DNA was extracted from laryngeal swabs with QIAAMP DNA MINI KIT™ (QIAGEN®, Hilden, Germany) according to manufacturer's instructions. A real-time PCR for *M. hyopneumoniae* was performed using primers previously described by Dubosson et al. (2004). Briefly, a total volume of 25 μL were used for the assay and results were analyzed with Fast Software 2.3 (Applied Biosystems™, Foster City, CA, USA). A Ct value of 39.5 was used as a cut-off for detection of samples positive for *M. hyopneumoniae*.

2.6. Statistical analysis

The *M. hyopneumoniae* prevalence was estimated at each one of the 12 samplings in gilts. Prevalence estimation was based on the number of positive gilts for *M. hyopneumoniae* by real-time PCR over the total number of gilts sampled. Multiple comparison tests with Bonferroni correction were performed in R v3.2 (R Core Team, 2015) to compare the prevalence of *M. hyopneumoniae* by real-time PCR at the various samplings.

3. Results

3.1. Mycoplasma hyopneumoniae detection in laryngeal swabs in gilts

Prior to the transference of gilts to the farrowing unit at 150 doa, a proportion of gilts in this study were culled based on the fact that they did not meet the genetic selection criteria. A total of 36 (45%) gilts were culled or did not live after selection at 150 doa. Therefore, 55% (44/80) of the gilts originally enrolled in the study were sampled in all 12 samplings events. Thus, results are presented for 44 gilts that were followed from beginning to end of the study, totaling 528 samples.

The prevalence of *M. hyopneumoniae* positive gilts at all samplings is shown in Fig. 2. *Mycoplasma hyopneumoniae* was not detected in gilts during the first three samplings, at 20, 50, and 80 doa. The first detection of *M. hyopneumoniae* occurred in 5/44 gilts at 110 doa, with a prevalence of 11.4%. A significant increase in *M. hyopneumoniae* prevalence was observed from 110 doa (11.4%) to 140 doa (36.4%; $p < 0.05$), and a gradual decrease in prevalence was observed over-time thereafter, although the *M. hyopneumoniae* prevalence did not reach 0 in any of the subsequent samplings (110–326/358 doa). No statistical differences were observed when comparing the *M. hyopneumoniae* prevalence in gilts at all subsequent samplings. The prevalence of *M. hyopneumoniae* did not exceed 36.4% at any sampling.

The pattern of *M. hyopneumoniae* detection in each gilt was assessed, and a schematic distribution is presented in Fig. 3. Eight gilts (18.2%) were continuously detected negative for *M. hyopneumoniae* at all samplings in the study, 34.1% (15/44) were detected positive in one sampling event, 22.7% (10/44) were positive twice, 20.4% (9/44) were positive three times, 2.3% (1/44) were positive four times, and 2.3% (1/44) were positive five times throughout the study. *Mycoplasma hyopneumoniae* was detected in 27.3% of gilts (12/44) more than once in intermittent samplings.

3.2. Piglets laryngeal swabs

A total of 220 piglets born to the 44 dams in the study were sampled one day prior to weaning. All piglets resulted negative for *M. hyopneumoniae* detection in laryngeal swabs by PCR (data not shown).

4. Discussion

A longitudinal study performed to assess the *M. hyopneumoniae* natural detection pattern *in vivo* in 44 replacement gilts is described here. Laryngeal swabs were collected every 30 days, from 20 doa to one day prior to the weaning event (326–358 doa), and a total of 528 samples were processed for *M. hyopneumoniae* detection using real-time PCR. Results showed that bacterial detection started at 110 doa and a significant increase in prevalence occurred at 140 doa compared to

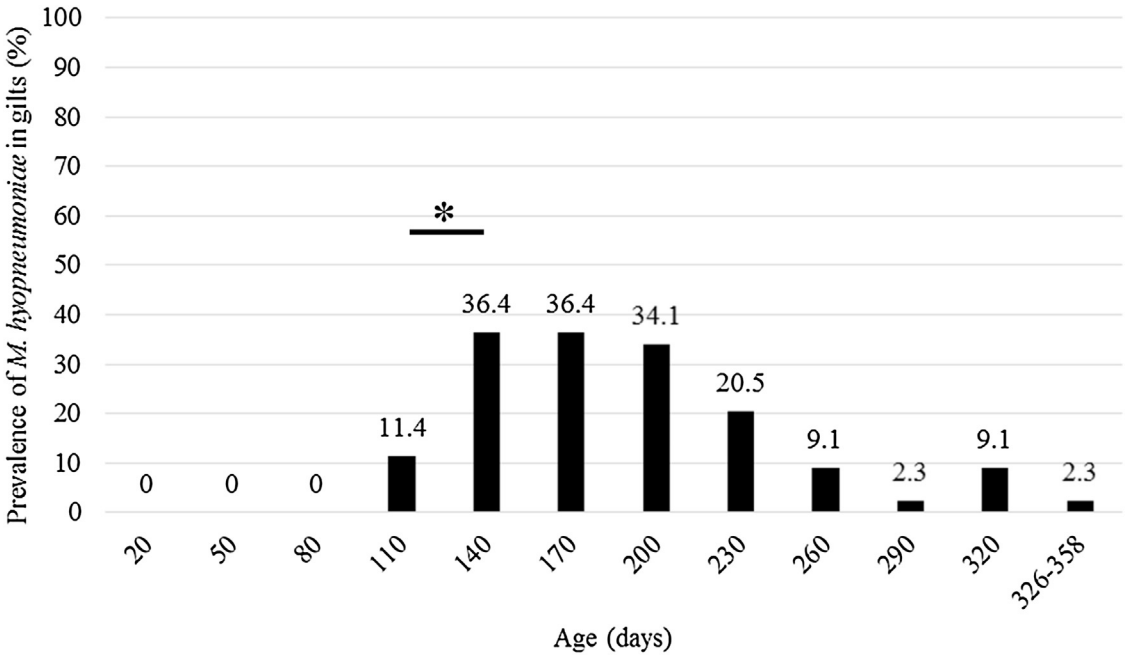

Fig. 2. *Mycoplasma hyopneumoniae* prevalence in gilts (n = 44) by real-time PCR from 20 days of age (doa) up to one day prior to weaning (326–358 doa). Gilts were sampled via laryngeal swabs every 30 days for *M. hyopneumoniae* detection by real-time PCR. Asterisks represent statistical difference (p-value < 0.05) between the *M. hyopneumoniae* prevalence at subsequent ages. The *M. hyopneumoniae* prevalence at all samplings was compared using multiple comparison tests and Bonferroni adjustments. Prevalence is shown in the graph at each sampling point.

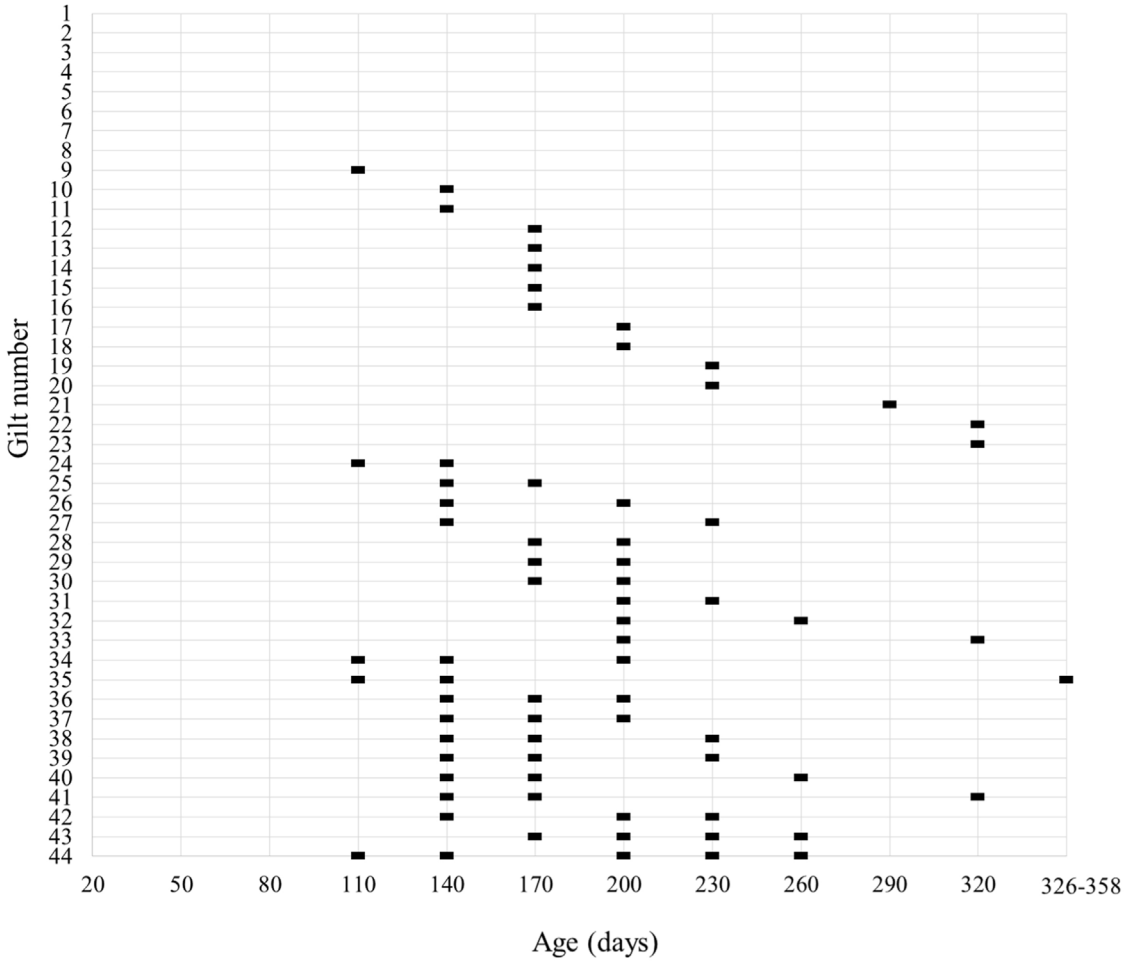

Fig. 3. Schematic representation of gilts (n = 44) according to the number of positive detection of *M. hyopneumoniae* by real-time PCR at all samplings. Laryngeal swabs were collected every 30 days from 20 doa to one day prior to weaning (326–358 doa). Each black box represents one sampling event for which the gilt was detected positive for *M. hyopneumoniae*.

previous sampling. The *M. hyopneumoniae* prevalence remained above 20% from 140 to 230 doa. Although low after 230 doa, the *M. hyopneumoniae* prevalence did not reach 0% at any sampling after 110 doa. It is important to note that *M. hyopneumoniae* was not detected in piglets born to the selected gilts that were sampled prior to weaning, while still on their biological dams. Also, the *M. hyopneumoniae* detection pattern in gilts was evaluated in this study, providing information about the duration of bacterial detection dynamics, potentially including the acute and chronic phases of infection.

Mycoplasma hyopneumoniae was not detected in any of the gilts at 20, 50 and 80 doa even though animals were known to be sourced from an endemically infected farm. Detection of *M. hyopneumoniae* started at 110 doa with a significant increase in prevalence at 140 doa in gilts. An increase in the transmission by direct contact with pigs from different farms is unlikely to explain the increase in prevalence, since gilts were housed only with gilts from the same farm and the same batch. It is speculated that gilts were already colonized with *M. hyopneumoniae*, as they originated from an endemically infected farm, but it was not detected while young. Nasal swabs have been widely used for *M. hyopneumoniae* detection, especially for young piglets. However, the bacterium shedding in nasal cavity may be intermittent (Pieters and Pijoan, 2006), affecting the test sensitivity. On the other hand, tracheobronchial (Kurth et al., 2002; Fablet et al., 2010) and laryngeal (Sievers et al., 2015; Pieters et al., 2017) sites appear to be the most sensitive to detect *M. hyopneumoniae* colonization in post-weaned and finishing pigs. Despite the apparent high sensitivity of laryngeal swabs, it can be proposed that a critical number of *M. hyopneumoniae* organisms is needed for pathogen detection *in vivo*. It seems that the bacterium does not reach the larynx until proliferation in the lower respiratory tract is sufficient, as infection with *M. hyopneumoniae* is slow, and the bacterial load in the larynx could remain undetected in early natural infections.

Despite knowing that *M. hyopneumoniae* shedding and transmission may occur for up to 214 days in experimental infections (Pieters et al., 2009), the results of this study suggest that in natural infections, gilts are positive for *M. hyopneumoniae* for one to three months, but occasionally, long-term detection (for four or five months) may occur. In experimental conditions, it is possible that the *M. hyopneumoniae* shedding is longer due to the fact that pigs are inoculated intratracheally with a high bacterial concentration. On the other hand, in natural infections, the main transmission route is nose-to-nose contact and the bacterial load may be lower. Also, it is important to note that detection of *M. hyopneumoniae* in this study was only assessed in live pigs, opposed to the most sensitive transmission experiments and post-mortem detection performed by Pieters et al. (2009).

The lack of *M. hyopneumoniae* detection throughout the study in 18.2% of gilts could be explained by the low transmission rate of the bacterium (Meyns et al., 2004; Pieters et al., 2010; Villarreal et al., 2011). This suggests that not all gilts become infected and are detected positive at the same time, and that negative gilts, still susceptible to *M. hyopneumoniae* infection, remain within the group even in endemically infected herds. The subpopulation of uninfected gilts potentially perpetuate *M. hyopneumoniae* infections by allowing constant pathogen circulation among pigs. Subpopulations were initially described in PRRSv endemic herds by Dee et al. (1996), where a subset of 20–25% negative dams were detected by serological evaluation during a six-month period. In this study, a similar proportion of *M. hyopneu-*

moniae negative gilts was detected even though gilts were followed during one year and tested via real-time PCR. To the best of our knowledge this is the first study suggesting the existence of subpopulations for *M. hyopneumoniae* in positive reproductive herds. Based on these results, the use of an acclimatization protocol can be recommended, in order to expose all gilts early in life, to guarantee they will be healed up and ceased shedding prior to their first farrowing (Pieters and Fano, 2016), even for gilts from *M. hyopneumoniae* positive herds introduced in positive farms. Also, it could be possible that *M. hyopneumoniae* shedding occurs mainly in the acute phase of infection and could not be detected when gilts are chronically infected (Pieters et al., 2009), which could have happened in this study.

Intermittent detections of *M. hyopneumoniae* were observed in 27.3% of gilts in this study. This might have occurred due to the failure to detect *M. hyopneumoniae* positive gilts, as the sensitivity of laryngeal swabs in naturally infected pigs is not perfect (Roos et al., 2016). Another potential explanation could be reinfection with different strains of *M. hyopneumoniae*. It is already known that different variants circulate among farms (Vranckx et al., 2011; Dos Santos et al., 2015; Pantoja et al., 2016) and cross protection among strains with different virulence may not occur (Villarreal et al., 2009). In this study, it would have been informative to characterize the *M. hyopneumoniae* variants detected in gilts over time, however the DNA concentration was insufficient for testing in most samples.

Despite the fact that all gilts were vaccinated twice for *M. hyopneumoniae* and the last dose was administrated at 150 doa, vaccination did not prevent infection with the bacterium, as previously demonstrated (Meyns et al., 2006; Pieters et al., 2010; Villarreal et al., 2011). On the other hand, vaccination could have had an effect potentially decreasing the infectious pressure in the group, as it is recognized that vaccinated and infected pigs exhibit a lower bacterial load and fewer clinical signs, compared with non-vaccinated counterparts (Vranckx et al., 2012; Woolley et al., 2014; Fano and Cline, 2016).

Antibiotic treatment may reduce the load of *M. hyopneumoniae* in the respiratory tract (Vicca et al., 2005), improve performance parameters, and decrease lesions and clinical signs of *M. hyopneumoniae* infection (Vicca, 2005). However, antibiotic treatments are not capable to eliminate the *M. hyopneumoniae* from the respiratory tract (Le Carrou et al., 2006; Painter et al., 2012). Potentially, the use of antibiotics in gilts in this study could have influenced the shedding of *M. hyopneumoniae*, and therefore its detection in laryngeal swabs. Nevertheless, an increase of positive gilts from 110 doa to 170 doa was observed when gilts were being medicated. For this reason, it can be hypothesized that the antibiotic treatment may have had minor influence in the *M. hyopneumoniae* detection in gilts in this study.

In this study, *M. hyopneumoniae* sow-to-piglet transmission was not detected, although one dam was positive for this bacterium at the same time when piglets were sampled. Previous studies have shown that in segregated systems a low proportion of young piglets are detected positive for *M. hyopneumoniae* and an abrupt increase in the number of colonized pigs at late age can be observed (Sibila et al., 2004; Giacomini et al., 2016).

5. Conclusions

Under the conditions of this study, the *M. hyopneumoniae in vivo* detection pattern showed that in natural infections gilts may be detected positive for *M. hyopneumoniae* for one to three months, and long-term detection may occur, although it may be less frequent. Moreover, the lack of *M. hyopneumoniae* detection in a considerable proportion of gilts indicated the existence of subpopulations within positive herds.

Competing interests

The authors declare that they have no competing interests.

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References

Calsamiglia, M., Pijoan, C., 2000. Colonisation state and colostral immunity to *Mycoplasma hyopneumoniae* of different parity sows. Vet. Rec. 146, 530–532.
Calsamiglia, M., Pijoan, C., Bosch, G.J., 1999. Profiling *Mycoplasma hyopneumoniae* in farms using serology and a nested PCR technique. Swine Health Prod. 7, 263–268.
Clark, L., Armstrong, C., Scheidt, A., Van Alstine, W., 1993. The effect of Mycoplasma hyopneumoniae infection on growing pigs with or without environmental constrains. Swine Health Prod . 6, 10–14.
Dee, A., Joo, H.S., Henry, S., Tokach, L., Park, B.K., Molitor, T., Pijoan, C., 1996. Detecting subpopulations after PRRS virus infection in large breeding herds using multiple serologic tests. J. Swine Health Prod. 4, 181–184.
Dos Santos, L.F., Sreevatsan, S., Torremorell, M., Moreira, M., Sibila, M., Pieters, M., 2015. Genotype distribution of Mycoplasma hyopneumoniae in swine herds from different geographical regions. Vet. Microbiol. 175, 374–381.
Dubosson, C.R., Conzelmann, C., Miserez, R., Boerlin, P., Frey, J., Zimmermann, W., Hani, H., Kuhnert, P., 2004. Development of two real-time PCR assays for the detection of Mycoplasma hyopneumoniae in clinical samples. Vet. Microbiol. 102, 55–65.
Fablet, C., Marois, C., Kobisch, M., Madec, F., Rose, N., 2010. Estimation of the sensitivity of four sampling methods for Mycoplasma hyopneumoniae detection in live pigs using a Bayesian approach. Vet. Microbiol. 143, 238–245.
Fano, E., Cline, G., 2016. Mycoplasma hyopneumoniae microbial load and histological lesion reduction in piglets vaccinated with MycoFLEX®. In: Proceedings of the International Pig Veterinary Society Congress. Dublin. pp. 237.
Fano, E., Pijoan, C., Dee, S., 2005. Dynamics and persistence of Mycoplasma hyopneumoniae infection on pigs. Can. J. Vet. Res. 69, 223–228.
Giacomini, E., Ferrari, N., Pitozzi, A., Remistani, M., Giardiello, D., Maes, D., Alborali, G.L., 2016. Dynamics of Mycoplasma hyopneumoniae seroconversion and infection in pigs in the three main production systems. Vet. Res. Commun. 40, 81–88.
Goodwin, R.F.W., Pomeroy, A.P., Whittlestone, P., 1965. Production of enzootic pneumonia in pigs with a mycoplasma. Vet. Rec. 77, 1247–1249.
Kurth, K.T., Hsu, T., Snook, E.R., Thacker, E.L., Thacker, B.J., Minion, F.C., 2002. Use of a Mycoplasma hyopneumoniae nested polymerase chain reaction test to determine the optimal sampling sites in swine. IJ. Vet. Diagn. Invest. 14, 463–469.
Le Carrou, J., Laurentie, M., Kobisch, M., Gautier-Bouchardon, A.V., 2006. Persistence of Mycoplasma hyopneumoniae in experimentally infected pigs after marbofloxacin treatment and detection of mutations in the parC gene. Antimicrob. Agents Chemother. 50, 1959–1966.
Maes, D., Segales, J., Meyns, T., Sibila, M., Pieters, M., Haesebrouck, F., 2008. Control of Mycoplasma hyopneumoniae infections in pigs. Vet. Microbiol. 126, 297–309.
Mare, C.J., Switzer, W.P., 1965. New Species: *mycoplasma hyopneumoniae*; a causative agent of virus pig pneumonia. Vet. Med. Small Anim. Clin. 60, 841–846.
Meyns, T., Maes, D., Dewulf, J., Vicca, J., Haesebrouck, F., de Kruif, A., 2004. Quantification of the spread of *Mycoplasma hyopneumoniae* in nursery pigs using transmission experiments. Prev. Vet. Med. 66, 265–275.
Meyns, T., Dewulf, J., de Kruif, A., Calus, D., Haesebrouck, F., Maes, D., 2006. Comparison of transmission of Mycoplasma hyopneumoniae in vaccinated and non-vaccinated populations. Vaccine 24, 7081–7086.
Painter, T., Kuhn, M., Wolff, T., Pieters, M., Senn, M., 2012. Efficacy and duration of infection study for RespiSure® and Draxxin® against a *Mycoplasma hyopneumoniae* challenge in swine. In: Proceedings of the International Pig Veterinary Society Congress. Jeju, Korea. pp. 207.
Pantoja, L.G., Pettit, K., Dos Santos, L.F., Tubbs, R., Pieters, M., 2016. *Mycoplasma hyopneumoniae* genetic variability within a swine operation. J. Vet. Diagn. Invest. 28, 175–179.
Pieters, M., Fano, E., 2016. *Mycoplasma hyopneumoniae* management in gilts. Vet. Rec. 178, 122–123.
Pieters, M., Pijoan, C., 2006. Detection pattern of *Mycoplasma hyopneumoniae* DNA in nasal swabs. In: Proceedings of the International Pig Veterinary Society Congress. Copenhagen, Denmark. pp. 209.
Pieters, M., Pijoan, C., Fano, E., Dee, S., 2009. An assessment of the duration of *Mycoplasma hyopneumoniae* infection in an experimentally infected population of pigs. Vet. Microbiol. 134, 261–266.
Pieters, M., Fano, E., Pijoan, C., Dee, S., 2010. An experimental model to evaluate *Mycoplasma hyopneumoniae* transmission from asymptomatic carriers to unvaccinated and vaccinated sentinel pigs. Can. J. Vet. Res. 74, 157–160.
Pieters, M., Daniels, J., Rovira, A., 2017. Comparison of sample types and diagnostic methods for in vivo detection of *Mycoplasma hyopneumoniae* during early stages of infection. Vet. Microbiol. in press.
R Core Team, 2015. R: A Language and Environment for Statistical Computing [Computer Software]. R Foundation for Statistical Computing, Vienna, Austria (Available from http://www.r-project.org/).
Roos, L.R., Fano, E., Homwong, N., Payne, B., Pieters, M., 2016. A model to investigate the optimal seeder-to-naïve ratio for successful natural *Mycoplasma hyopneumoniae* gilt exposure prior to entering the breeding herd. Vet. Microbiol. 184, 51–58.
Sibila, M., Calsamiglia, M., Vidal, D., Badiella, L., Aldaz, A., Jensen, J.C., 2004. Dynamics of *Mycoplasma hyopneumoniae* infection in 12 farms with different production

systems. Can. Can. J. Vet. Res. 68, 12–18.
Sievers, C., Dalquist, L., Sponheim, A., Fano, E., Wetzell, T., 2015. Comparison of antemortem sampling procedures to post-mortem bronchial sampling for the detection of *Mycoplasma hyopneumoniae* by PCR. In: Proceedings of the Annual Meeting of the American Association of Swine Veterinarians. Orlando, FL. pp. 115–116.
Thacker, E.L., Minion, F.C., 2012. Mycoplasmosis. In: Zimmermann, J.J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G.W. (Eds.), Diseases of Swine, tenth ed. Wiley-Blackwell Ames, pp. 779–797.
Vangroenweghe, F., Karriker, L., Main, R., Christianson, E., Marsteller, T., Hammen, K., Bates, J., Thomas, P., Ellingson, J., Harmon, K., Abate, S., Crawford, K., 2015. Assessment of litter prevalence of *Mycoplasma hyopneumoniae* in preweaned piglets utilizing an antemortem tracheobronchial mucus collection technique and a real-time polymerase chain reaction assay. J. Vet. Diagn. Invest. 27, 606–610.
Vicca, J., Maes, D., Jonker, L., de Kruif, A., Haesebrouck, F., 2005. The efficacy of tylosin premix for the treatment and control of *Mycoplasma hyopneumoniae* infections. Vet. Rec. 156, 606–610.
Vicca, J., 2005. Virulence and Antimicrobial Susceptibility of *Mycoplasma Hyopneumoniae* Isolates from Pigs. PhD Thesis. Faculty of Veterinary Medicine, Ghent Universitypp. 219 ISBN 90-5864-086-8.
Villarreal, I., Maes, D., Meyns, T., Gebruers, F., Calus, D., Pasmans, F., Haesebrouck, F., 2009. Infection with a low virulent *Mycoplasma hyopneumoniae* isolate does not protect piglets against subsequent infection with a highly virulent *M. hyopneumoniae* isolate. Vaccine 1875–1879.
Villarreal, I., Meyns, T., Dewulf, J., Vranckx, K., Calus, D., Pasmans, F., Haesebrouck, F., Maes, D., 2011. The effect of vaccination on the transmission of *Mycoplasma hyopneumoniae* in pigs under field conditions. Vet. J. 188, 48–52.
Vranckx, K., Maes, D., Calus, D., Villarreal, I., Pasmans, F., Haesebrouck, F., 2011. Multiple-locus variable-number tandem-repeat analysis is a suitable tool for differentiation of *Mycoplasma hyopneumoniae* strains without cultivation. J. Clin. Microbiol. 49, 2020–2023.
Vranckx, K., Maes, D., Marchioro, S.B., Villarreal, I., Chiers, K., Pasmans, F., Haesebrouck, F., 2012. Vaccination reduces macrophage infiltration in bronchus-associated lymphoid tissue in pigs infected with a highly virulent *Mycoplasma hyopneumoniae* strain. Vet. Res. 8, 1–7.
Woolley, L.K., Fell, S.A., Gonsalves, J.R., Raymond, B.B.A., Collins, D., Kuit, T.A., Walker, M.J., Djordjevic, S.P., Eamens, G.J., Jenkins, C., 2014. Evaluation of recombinant *Mycoplasma hyopneumoniae* P97/P102 paralogs formulated with selected adjuvants as vaccines against mycoplasmal pneumonia in pigs. Vaccine 32, 4333–4341.

4. CAPÍTULO III – SEGUNDO ARTIGO CIENTÍFICO

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Infection dynamics and genetic variability of *Mycoplasma hyopneumoniae* in self-replacement gilts

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ABSTRACT

The aim of this study was to assess the longitudinal pattern of *M. hyopneumoniae* detection in self-replacement gilts at various farms and to characterize the genetic diversity among samples. A total of 298 gilts from three *M. hyopneumoniae* positive farms were selected at 150 days of age (doa). Gilts were tested for *M. hyopneumoniae* antibodies by ELISA, once in serum at 150 doa and for *M. hyopneumoniae* detection in laryngeal swabs by real time PCR two or three times. Also, 425 piglets were tested for *M. hyopneumoniae* detection in laryngeal swabs. A total of 103 samples were characterized by Multiple Locus Variable-number tandem repeats Analysis. Multiple comparison tests were performed and adjusted using Bonferroni correction to compare prevalences of positive gilts by ELISA and real time PCR. Moderate to high prevalence of *M. hyopneumoniae* in gilts was detected at 150 doa, which decreased over time, and different detection patterns were observed among farms. Dam-to-piglet transmission of *M. hyopneumoniae* was not detected. The characterization of *M. hyopneumoniae* showed 17 different variants in all farms, with two identical variants detected in two of the farms. ELISA testing showed high prevalence of seropositive gilts at 150 doa in all farms. Results of this study showed that circulation of *M. hyopneumoniae* in self-replacement gilts varied among farms, even under similar production and management conditions. In addition, the molecular variability of *M. hyopneumoniae* detected within farms suggests that in cases of minimal replacement gilt introduction bacterial diversity maybe farm specific.

1. Introduction

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is the primary pathogen of enzootic pneumonia (Maes et al., 2008), a chronic bronchopneumonia which has been associated with economic losses due to reduced daily weight gain and feed efficiency, increased mortality and production costs due to medication and vaccination (Thacker and Minion, 2012). Additionally, *M. hyopneumoniae* predisposes pigs to infections with viruses and other bacteria (Thacker, 2006), playing an important role in the porcine respiratory disease complex (PRDC; Dee, 1996; Thacker and Minion, 2012).

Transmission of *M. hyopneumoniae* usually occurs by direct contact between susceptible and infectious pigs, although indirect contact also has importance in the infection dynamics of this bacterium (Fano et al., 2005). Disease presentation may vary with the production system. Sibila et al. (2004) and Giacomini et al. (2016) observed that in single-site herds the infection seems to occur early, due the contact between susceptible piglets and colonized dams, and increases progressively with age. On the other hand, in segregated production systems the prevalence of *M. hyopneumoniae* increases towards the growing-finishing phase and pigs may develop enzootic pneumonia in the fattening phase. However, Villarreal et al. (2010) observed prevalences up to 22.1% of *M. hyopneumoniae* positive weaned piglets regardless the type of production system.

It has been suggested that young parity sows pose a greater risk to transmit *M. hyopneumoniae* to their offspring compared to older parity sows (Calsamiglia and Pijoan, 2000; Fano et al., 2006) and this could help disseminate the bacterium in farms (Pijoan, 2003). Pieters et al. (2014) observed that dams which shed *M. hyopneumoniae* during the lactation period influenced the probability of the piglets to become colonized at weaning, indicating the role of vertical transmission (sow-to-piglet) in *M. hyopneumoniae* infection dynamics, as others have also suggested (Clark et al., 1991; Nathues et al., 2013). Despite that, only a small proportion of piglets may be colonized in the first week of life

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(Sibila et al., 2007), and a variable number of piglets may become colonized prior to weaning (Fano et al., 2007; Villarreal et al., 2010; Pieters et al., 2014). Moreover it has been suggested that *M. hyopneumoniae* colonization in weaned piglets affects the severity of the disease and frequency of affected lungs at the slaughterhouse (Fano et al., 2007).

Dam replacement in reproductive herds, in order to maintain a young and highly productive sow farm, can be performed through external purchase or through self-replacement of gilts (Furtado et al., 2006). The purchase of gilts is necessary when genetic improvement is required, and should be performed from similar or higher health status herds (Maes et al., 2008), but may have unwanted consequences based on pathogen colonization and status of the incoming gilts as well as the recipient dams. On the other hand, the sanitary risks in self-replacement systems are lower (Furtado et al., 2006; Maes et al., 2008; Thacker and Minion, 2012), as gilts from the same farm are used, which does not represent a new introduction. Thus, this practice provides a stable herd immunity when compared with farms that purchase breeding gilts (Maes et al., 2008).

Self-replacement gilts may represent up to 25% of the reproductive herd in a pig farm at a given point of time (Furtado et al., 2006), which is an important segment of the herd population potentially affecting *M. hyopneumoniae* transmission. However, to date, little information is available on the dynamics and duration of *M. hyopneumoniae* infection in self-replacement gilts originated from positive farms. Therefore, the objective of this study was to assess the pattern of *M. hyopneumoniae* detection in self-replacement gilts from positive farms, and to characterize its genetic diversity within and among farms.

2. Materials and methods

2.1. Ethics statement

Farms involved in the study followed their own management practices. Study procedures were conducted under the approval of the Institutional Animal Care and Use Committee of the Federal University of Rio Grande do Sul, Brazil.

2.2. Herds

Three multiplier pig farms (A, B and C), from three different production companies, located in the three largest pig producer states in Brazil, namely Minas Gerais, Santa Catarina, and Rio Grande do Sul, were conveniently selected for this study. The farms were chosen based on the following criteria: 1) All herds were multi-site multipliers with at least 1500 sows located in geographic regions of high pig density; 2) Herds were *M. hyopneumoniae* positive based on respiratory clinical signs, diagnostic history, and the presence of medium to high prevalence of lung lesions at slaughter, and 3) Farms produced their own replacement gilts (self-replacement). In Farm A, gilts were raised in the multiplier farm up to 21 days of age (doa), moved and housed in a nursery (located 1 kilometer apart) up to 63 doa, and transported to a finishing unit (located 1 kilometer apart from the nursery) where remained up to 150 doa, when they returned to the multiplier farm. In Farm B and C, gilts were weaned and remained in the multiplier farm up to 63 doa, when they were moved and housed in a finishing unit (500 m apart from the multiplier B and 700 m from the multiplier C) up to 150 doa, at which age they returned to the multiplier farm. Farms A and B did not have history of introduction of other pigs. Farm C recorded the introduction of 74 *M. hyopneumoniae* negative gilts five months before the beginning of this study. Herd size, production system, and vaccination protocol for *M. hyopneumoniae* from the three farms are presented in Table 1.

Table 1
Characteristics of the three *Mycoplasma hyopneumoniae* positive multiplier pig farms selected for the study.

Farm	Location (state)	Herd Size (number of sows)	Production System (sites)	Vaccination against *M. hyopneumoniae* (doa)
A	Minas Gerais	3700	3	21, 42, 150[a]
B	Santa Catarina	2200	2	21, 42, 150[a]
C	Rio Grande do Sul	1500	2	21, 42, 150[b]

[a] Vaccination with commercial *M. hyopneumoniae* bacterin (PCV-M® – MSD Saúde Animal).
[b] Vaccination with commercial *M. hyopneumoniae* bacterin (M + Pac® – MSD Saúde Animal).

2.3. Experimental design

Approximately 100 gilts were randomly selected per farm at 150 doa, based on a low *M. hyopneumoniae* prevalence, a 95% confidence interval, and the fact that some gilts would be culled prior to their first service. Thus, a total of 298 gilts were enrolled in this study. Ninety-five gilts were selected in Farm A, 103 gilts in Farm B, and 100 gilts in Farm C. All gilts were ear tagged and sampled two (Farm A) or three times (Farm B and C) in a longitudinal fashion, from puberty induction (150 doa) through mid-gestation (270 doa), to first farrowing, lactation or second breeding (330 doa). Five piglets born to each selected gilts were also sampled one day prior to weaning (20 doa) to evaluate vertical transmission of *M. hyopneumoniae*, totaling 425 piglets (200 in Farm A, 110 in Farm B, and 115 in Farm C). Cross-fostering was not allowed during the study to guarantee sampling piglets of their biological dams. A graphic representation of the experimental design is shown in Fig. 1.

2.4. Sample collection

Laryngeal swabs were obtained from gilts and piglets, as previously described by Pieters et al. (2017). Briefly, a mouth gag and a laryngoscope were used for the introduction of a sterile rayon swab (Laborclin®, Pinhais, PR, Brazil) in the mouth cavity until they reached the larynx, where swabs were pressed against the structure wall. All swabs were identified, refrigerated, and delivered to the laboratory where they were frozen at −20 °C until analysis. Blood samples were collected from all gilts from the cranial vena cava prior the third vaccination for *M. hyopneumoniae*. Blood collection was performed using vacuum tubes (Labor Import, Osasco, SP, Brazil) and sterile needles (Labor Import, Osasco, SP, Brazil) as described by Ramirez and Karriker (2012). Serum was obtained from blood samples using a centrifuge (Spintech, CTP-SI, Porto Alegre, RS, Brazil) at 3200 rpm for three minutes. Serum was aliquoted and stored at −20 °C until analysis.

2.5. Sample processing and testing

Real time PCR for *M. hyopneumoniae*: Laryngeal swabs were processed for DNA extraction with QIAAMP DNA MINI KIT™ (QIAGEN®, Hilden, Germany), according to manufacturer's instructions. Two sets of primers (Invitrogen, Waltham, MA, USA) were synthesized from the whole genome analysis of *M. hyopneumoniae*, *M, hyorhinis* and *M. flocculare,* which the 50S ribosomal gene was selected, in a region specific for *M. hyopneumoniae*. The forward primer was 5′-CCCAGCAAAGTCAGGCTGAA-3′ (nucleotide positions 419–438) and the reverse primer was 5′-TGGGAATCAACCCCATTTTC-3′ (nucleotide positions 457–476). The sensitivity and specificity of the assay were analyzed using serial dilutions of the bacterium for sensitivity (95%) and a battery of bacterial strains including *M. hyopneumoniae* and other Mycoplasma species previously identified in swine for specificity

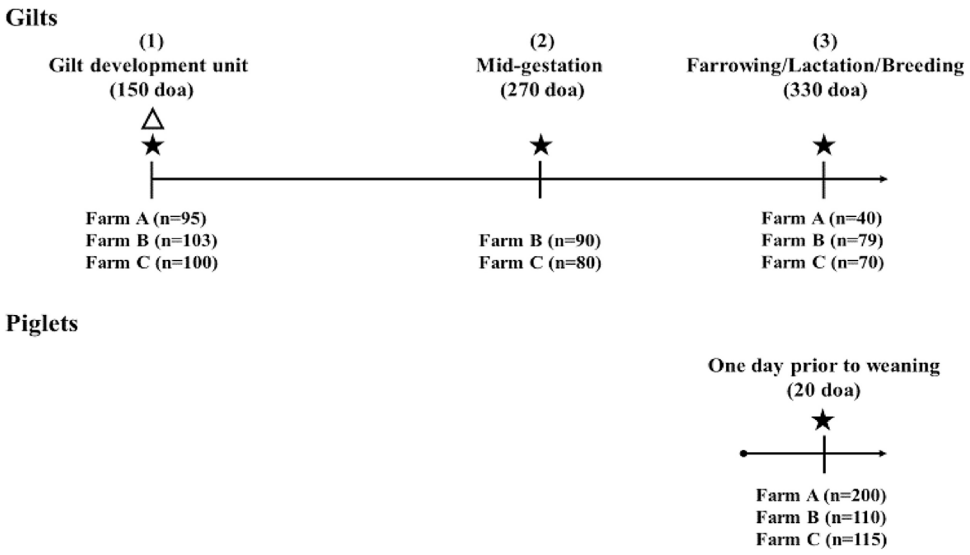

Fig. 1. Graphic representation of the study experimental design. A total of 298 gilts were selected from farms A, B and C for detection of *M. hyopneumoniae* in a longitudinal fashion. Collection of laryngeal swabs is represented as solid stars and blood sampling is represented as open triangle. Farm A: laryngeal swabs were collected in the first and third events for real time PCR testing, totaling 135 samples. Farms B and C: laryngeal samples were collected in the three events for real time PCR testing, totaling 272 and 250 samples per farm, respectively. A total of 425 piglets born to the selected gilts were sampled by laryngeal swabs one day prior to weaning to evaluate vertical transmission of *M. hyopneumoniae* by real time PCR testing.

(98%). Amplification was performed in a final volume of 25 μL, which consisted of 1 x PCR buffer 10 x concentrated (Invitrogen, Waltham, MA, USA), 1.5 mM $MgCl_2$ (Invitrogen, Waltham, MA, USA), 200 nM dNTP (Invitrogen, Waltham, MA, USA), 1 U *Taq* DNA polymerase (Invitrogen, Waltham, MA, USA), 19.05 μL nuclease free water (Applied Biosystems™, Foster City, CA, USA), 30 pmol of each primer, 20 pmol Taqman probe (5′-FAM-AACCCCAAAATGACC-BHQ1-3′; Applied Biosystems™, Foster City, CA, USA) and 1 μL of each sample. Reactions were performed in a thermocycler (Fast Real-time PCR System, Applied Biosystems™, Foster City, CA, USA) under the following conditions: denaturation at 95 °C for 5 min, 40 cycles of, denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 69 °C for 1 min. Amplifications were analyzed with Fast Software 2.3 (Applied Biosystems™, Foster City, CA, USA). In each plate, two positive (*M. hyopneumoniae* genetic material, primers and mix with reagents) and two negative controls (primers and mix with reagents, along with distilled water) were used. Samples from gilts were tested individually by real time PCR in duplicate, and samples from piglets were tested in pools of five within litter, unless piglets were born to positive dams, in which case they were tested individually, in duplicate. Samples were considered positives for real time PCR when Ct ≤ 39.5.

Multiple Locus Variable-number tandem repeats Analysis (MLVA): One hundred and three positive *M. hyopneumoniae* real time PCR samples with Ct values lower than 31 were characterized at the molecular level using MLVA typing. Briefly, two sets of primers of two VNTR loci P97 RR1 (*mhp* 138) and P146 RR3 (*mhp* 684) were used in this study and reactions were performed as previously described by Dos Santos et al. (2015). A highly characterized strain of *M. hyopneumoniae* (strain 232) was used as a positive control. The assay parameters and the algorithm used for analysis have been previously described (Dos Santos et al., 2015). Analysis was performed in Bionumerics version 7.0 (Applied Maths, East Flanders, Belgium).

Enzyme-linked immunosorbent assay (ELISA): The detection for *M. hyopneumoniae* antibodies was performed using a commercial ELISA test (Idexx Laboratories, Westbrook, ME, USA), following manufacturer's protocol. Samples were considered positive when S/P ratio ≥ 0.4, suspects when S/P ratio was above 0.3 and below 0.4, and negatives below 0.3.

2.6. Statistical analysis

The *M. hyopneumoniae* prevalence for each herd and sampling event was estimated based on the number of laryngeal swabs or serum detected positive for *M. hyopneumoniae* by PCR or ELISA, respectively. Multiple comparison (analysis of variance − ANOVA) tests with Bonferroni adjustment were performed in R v3.2 (R Core Team, 2015) to compare prevalences among sampling events at each farm for PCR, and ELISA results were compared among farms, as one sampling was performed. On each sampling event, the confidence interval was calculated (95%). Likewise, Agresti-Coull interval was calculated for prevalences equal to 0%. Statistical significance was considered when p-values were less than 0.05.

3. Results

3.1. Mycoplasma hyopneumoniae detection in laryngeal swabs in gilts

During the study, culling and mortality of gilts (approximately 25%) occurred between samplings, especially due to reproductive failure and stomach ulcers. Therefore, a total of 657 laryngeal swabs (135 in Farm A, 272 in Farm B, and 250 in Farm C) were obtained from gilts in all sampling events. The prevalence of *M. hyopneumoniae* by real time PCR in all farms at all samplings is presented in Fig. 2. In Farm A, a prevalence of 67.4% was observed in the first sampling, while no positive gilts were detected in the third sampling. In Farm B, statistical differences ($p < 0.05$) on *M. hyopneumoniae* prevalence were detected among all samplings, which decreased over time from the first (57.3%) to the second (14.4%) and third (1.3%) samplings. The Farm C showed a similar prevalence for *M. hyopneumoniae* in the first two samplings (47% and 47.5%, respectively) and a decrease from the second to the third sampling to 15.7% ($p < 0.05$).

A total of 189 gilts (40 in Farm A, 79 in Farm B and 70 in Farm C), that remained in the study up to completion, were analyzed individually to compare *M. hyopneumoniae* infection dynamic over time according to the real time PCR results (Table 2). In Farm A, 67.5% (27/40) of gilts sampled twice became negative for *M. hyopneumoniae* between the first and last samplings, and 32.5% (13/40) were detected negative at both samplings. In Farm B, 46.8% (37/79) of gilts were detected positive only in the first sampling and 39.2% (31/79) were detected negative in all samplings, representing the two most prevalent patterns. In Farm C, the two most prevalent patterns were: 25.7% (18/70) positive gilts in the second sampling and 22.9% (16/70) gilts detected positive in the first sampling.

3.2. Mycoplasma hyopneumoniae detection in laryngeal swabs in piglets

A total of 425 piglets were sampled one day prior to weaning in the three farms. All piglets resulted negative for *M. hyopneumoniae*

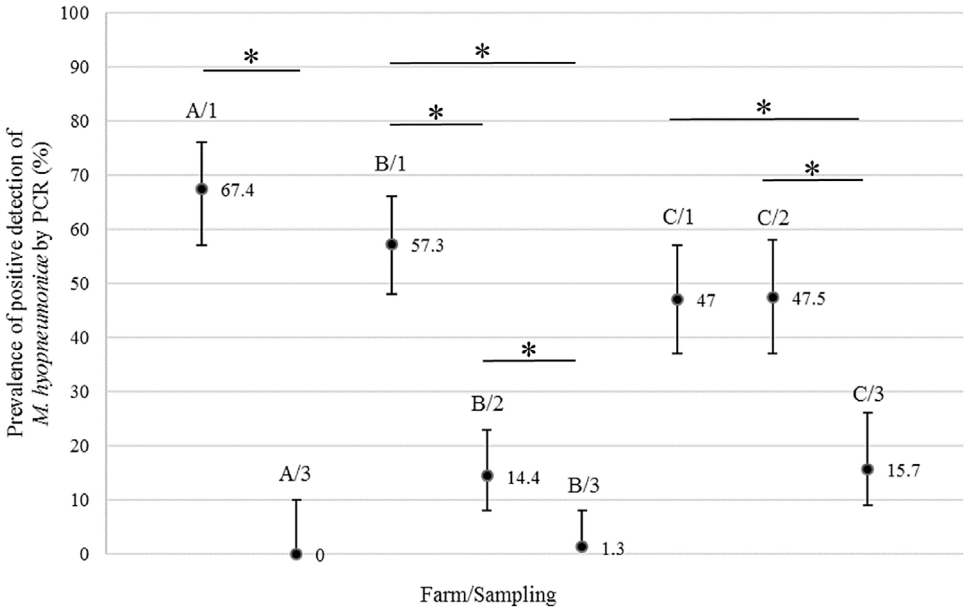

Fig. 2. *Mycoplasma hyopneumoniae* prevalence by real time PCR on three multiplier herds (A, B and C). Gilts were sampled in each farm two (Farm A) or three times (Farm B and Farm C) for *M. hyopneumoniae* detection in laryngeal swabs. A combination of a letter and a number were used to denote farm (A, B and C) and sampling (1, 2 and 3). Asterisks represent significant difference ($p < 0.05$) within the same farm based on multiple comparisons test and Bonferroni adjustment.

Table 2
Real time PCR status and prevalence of *Mycoplasma hyopneumoniae* of gilts at three different self-replacement farms.

Farm	*Mycoplasma hyopneumoniae* detection pattern			Prevalence% (positive/tested)
	First sampling	Second sampling	Third sampling	
A	+		−	67.5 (27/40)
	−		−	32.5 (13/40)
	+		+	0 (0/40)
	−		+	0 (0/40)
B	+	−	−	46.8 (37/79)
	−	−	−	39.2 (31/79)
	+	+	−	8.9 (7/79)
	−	+	−	3.8 (3/79)
	−	−	+	1.3 (1/79)
	+	+	+	0 (0/79)
	+	−	+	0 (0/79)
	−	+	+	0 (0/79)
C	−	+	−	25.7 (18/70)
	+	−	−	22.9 (16/70)
	−	−	−	20 (14/70)
	+	+	−	15.7 (11/70)
	−	+	+	5.7 (4/70)
	+	−	+	4.3 (3/70)
	−	−	+	4.3 (3/70)
	+	+	+	1.4 (1/70)

detection in laryngeal swabs by PCR (data not shown).

3.3. Multiple Locus Variable-number tandem repeats Analysis (MLVA)

A total of 103 samples obtained from all farms in the first sampling were used for MLVA characterization. Forty samples were obtained from Farm A, 37 from Farm B, and 26 from Farm C, which represented 42.1%, 35.9% and 26% of the total samples collected at the farms when gilts were 150 doa, respectively. A minimum spanning tree was prepared to demonstrate the genetic variation within and among farms (Fig. 3). Seventeen different variants were detected among farms. Two variants (3–17 and 3–24) were detected in Farm A as well as in Farm B. Six variants were detected in Farm A, 11 in Farm B, and two in Farm C, where the type 11–21 was the most prevalent. The locus P97 exhibited 5 types, ranging from 3 to 12 repeats, and 11 types of the P146 locus were detected, ranging from 14 to 45 repeats.

3.4. Mycoplasma hyopneumoniae detection in serum samples

A high prevalence of ELISA positive gilts for *M. hyopneumoniae* was observed in the three farms. Namely, 98.9% (94/95) in Farm A, 91.3% (94/103) in Farm B, 100% (100/100) in Farm C, with the seroprevalence between Farms B and C being statistically different ($p < 0.05$; Fig. 4).

4. Discussion

In this study, we investigated the pattern of *M. hyopneumoniae* detection in self-replacement gilts in three multiplier herds for a six-month period using real time PCR. Antibody detection was also performed in the same gilts at 150 doa, and real time PCR was used to detect *M. hyopneumoniae* in piglets one day prior to weaning.

Real time PCR results showed moderate to high prevalence of *M. hyopneumoniae* in gilts at 150 doa in all farms, which decreased between samplings. The prevalence of *M. hyopneumoniae* in gilts from three multiplier herds assessed in this study was similar at 150 doa, suggesting that gilts were already exposed to *M. hyopneumoniae* and were potentially shedding the bacterium. Sibila et al. (2004) and Giacomini et al. (2016) have suggested that pigs from segregated production systems present late *M. hyopneumoniae* infection, which might have occurred in the three farms assessed in this study. On the other hand, Fano et al. (2007) and Villarreal et al. (2010) showed that multisite farms may also present early *M. hyopneumoniae* infection, suggesting that the production system may not have a strong influence when piglets are colonized. Despite the prevalence of shedding gilts at 150 doa, a decrease of *M. hyopneumoniae* detection occurred until 330 doa, when prevalence ranged from 0 to 15.7%. It is important to note that Farm C showed a higher *M. hyopneumoniae* prevalence in gilts in the last sampling compared to Farms A and B, which could be related to the fact that negative *M. hyopneumoniae* gilts were introduced in Farm C five months prior to the beginning of the study. However, no samples were collected from the subset of negative gilts as part of this investigation. The introduction of replacement gilts could have led to herd instability, especially when gilts with different health status are introduced, as the case of *M. hyopneumoniae* negative gilts introduction in positive herds (Maes et al., 2008; Pieters and Fano, 2016). The detection of *M. hyopneumoniae* in naturally infected gilts from 20 to 326–358 doa has been previously reported by our research group (Takeuti et al., 2017), showing that the highest *M. hyopneumoniae*

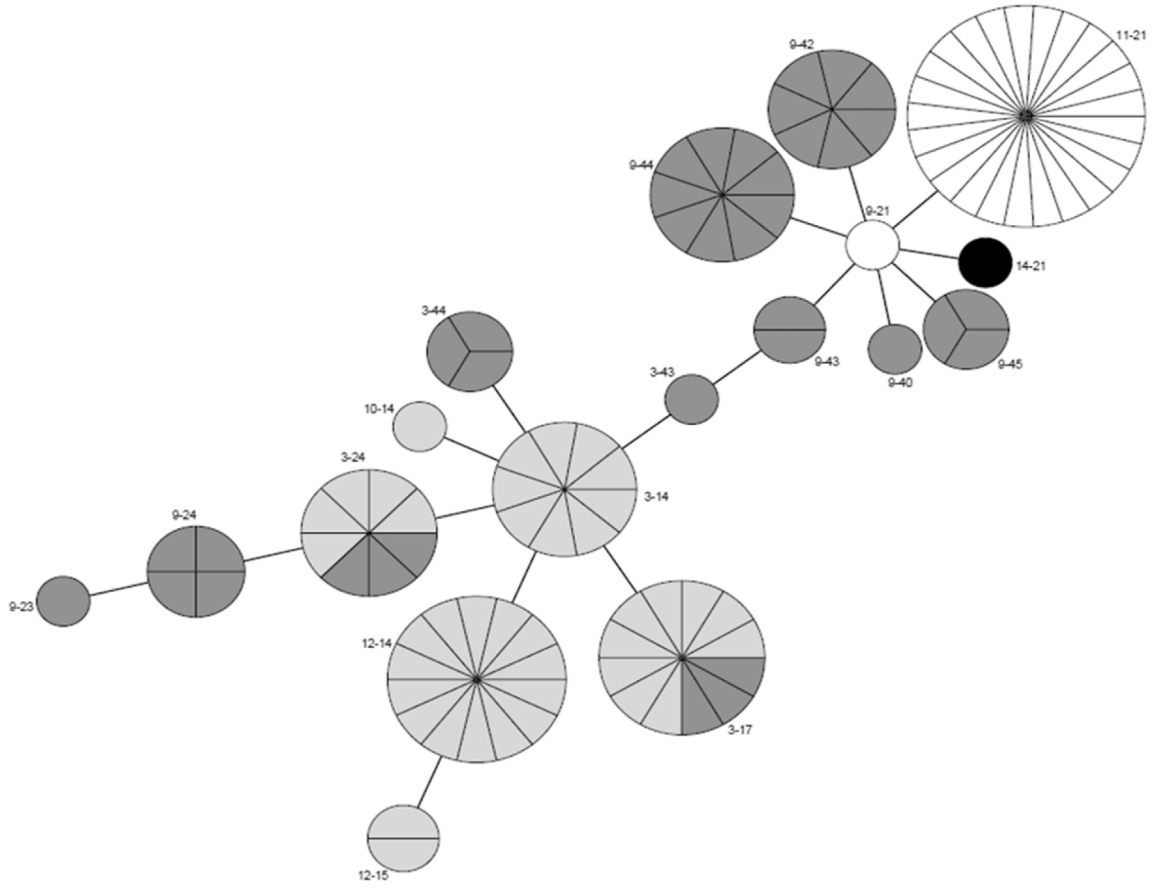

Fig. 3. Minimum spanning tree of *M. hyopneumoniae* samples characterized by MLVA. Samples from each farm are represented with three different colors: Farm A: light grey; Farm B: dark grey; Farm C: white. The U.S. 232 reference strain is represented by the black node. Six variants were detected in Farm A, 11 different variants in Farm B, and two in Farm C, where a high prevalence of one variant (11–21) was observed. A total of 17 variants were detected among farms and two variants (3–17 and 3–24) were found in two different farms (A and B).

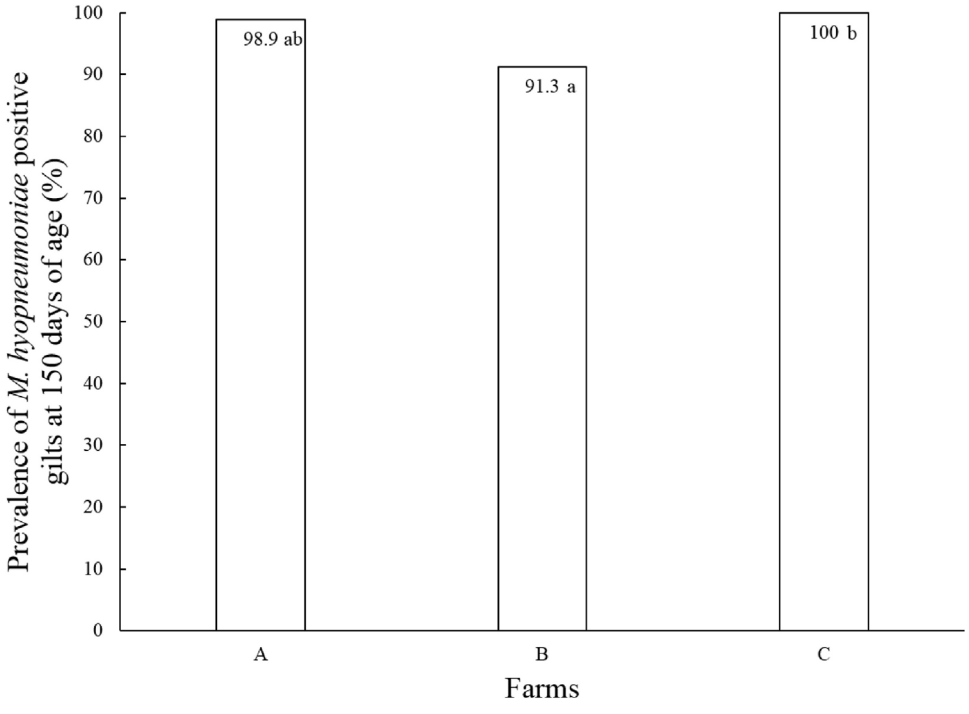

Fig. 4. Seroprevalence for *M. hyopneumoniae* in self-replacement gilts at 150 doa in Farms A, B and C. Different letters represent significant statistical difference based on multiple comparisons test and Bonferroni adjustment.

prevalence (36.4%) was detected in gilts at 140 and 170 doa, which is similar to results obtained in this study. However, a full profile of *M. hyopneumoniae* detection was not attempted in this investigation.

The detection pattern assessed in gilts that remained in the study up to completion was similar in all farms. The two most common patterns were: a single *M. hyopneumoniae* positive detection (in the first or second sampling), which represented 53.4% (101/189) of all gilts, and no positive detection, which was responsible for 30.7% (58/189) of all patterns. Similar results were observed by Takeuti et al. (2017) where 18.2% of gilts remained negative for *M. hyopneumoniae* by real time PCR during one year in a longitudinal study. The lack of positivity in 30.7% of the gilts in this study could have occurred due to the low transmission rate of *M. hyopneumoniae* (Meyns et al., 2004; Roos et al., 2016), suggesting that not all pigs become infected at the same time, which may result in subpopulations of susceptible gilts for a future exposure to the bacterium.

The genetic diversity of *M. hyopneumoniae* within farms has been described by MLVA (Nathues et al., 2011; Vranckx et al., 2011; Dos Santos et al., 2015; Pantoja et al., 2016; Michiels et al., 2017). Several studies have described a limited number of different variants in European and American herds (Vranckx et al., 2011; Pantoja et al., 2016). On the other hand, Nathues et al. (2011), Dos Santos et al. (2015) and Michiels et al. (2017) detected a great diversity of *M. hyopneumoniae* within farms. In this study, variability in the number of variants was identified between farms. In one farm, only two variants were detected using MLVA characterization. However, a large variability was observed in samples from the other two farms, which presented six and 11 different variants, indicating the circulation of a large diversity of *M. hyopneumoniae* in those farms. Among all 17 different variants that were detected in the three farms, interestingly two variants (3–17 and 3–24) were identified in two of the three farms in the study. Four variants identified in this study (10–14, 12–14, 12–15 and 11–21) were previously detected in the same state regions in Brazil in a previous investigation (Dos Santos et al., 2015). These results suggest that farms can share the same *M. hyopneumoniae* variants, and that variants can be identified in the same geographical regions over time. The genetic variability of *M. hyopneumoniae* in the same gilts over time could not be assessed in this study as the DNA concentration was insufficient for MLVA characterization in samples obtained in the second and third collections.

Limited variability in the locus P97 was observed, however, in P146 locus a high heterogeneity was detected, which also had a larger number of repeats. The same patterns were previously detected in samples obtained in Brazil by sequencing (De Castro et al., 2006) and MLVA (Dos Santos et al., 2015), which appear to be inherent to that region. All samples were analyzed following the same criteria and the peak with highest intensity was selected for analysis. However, the amplification of more than one peak in the electrophoresis analysis was observed in 35.9% (37/103) of samples (Farm A: 65%; 26/40; Farm B: 29.7%; 11/37; Farm C: 0%; 0/26), which could indicate the presence of multiple variants of *M. hyopneumoniae* in the same gilt, as previously suggested (Vranckx et al., 2011; Dos Santos et al., 2015). It is important to note that the presence of multiple variants of *M. hyopneumoniae* was not detected in Farm C, which also presented the lowest within farm variability. These results suggest that the presence of more than one variant in the same pig could occur due to the presence of multiple variants in the farm.

Antibody detection by ELISA is an important diagnostic tool for *M. hyopneumoniae* (Calsamiglia et al., 1999), but it does not allow to pinpoint when the animals become infected or to differentiate between natural infection and vaccination (Sibila et al., 2009). In this study, ELISA results showed high prevalence of seropositive gilts at 150 doa in all farms with high S/P values, with only 3.3% of gilts being negative or suspect for *M. hyopneumoniae*. Doubtlessly, vaccination could have played a role in the antibody detection in our study since gilts were vaccinated twice (21 and 42 doa) before the blood sampling. However, antibodies detected in our study could have been the result of natural *M. hyopneumoniae* infection, since Maes et al. (1999) observed that only 14.3% of piglets vaccinated at 7 and 21 doa against *M. hyopneumoniae* were seropositive at 110 doa, 89 days after most recent dose. In our study, gilts were vaccinated at 21 and 42 doa and the blood collection for testing was performed at 150 doa, 108 days after the most recent *M. hyopneumoniae* vaccination. Also, previous studies have shown antibody detection at the end of the finishing phase, which corresponds to the age of gilts in this study, may be due to natural infection (Sibila et al., 2009; Fraile et al., 2010).

Despite the proportion of seropositive gilts for *M. hyopneumoniae* at 150 doa observed in this study, not all seropositive gilts were positive by real time PCR, which could indicate a difference in sensitivity of ELISA when compared to PCR testing in the chronic phase of infection, since antibodies may take a long time to be produced, but remain detectable for a longer time (Morris et al., 1995), or could indicate the response to vaccination. The lack of positivity by real time PCR in seropositive gilts was observed even though samples were considered positive when Ct values were 39.5. The absence of detection by PCR could have occurred if those gilts were not shedding *M. hyopneumoniae* when they were sampled, as the acute phase of infection may have ended.

Several studies have shown that piglets may be colonized by *M. hyopneumoniae* during the lactation period and are weaned positive for this bacterium (Fano et al., 2007; Sibila et al., 2007; Villarreal et al., 2010; Pieters et al., 2014). However, no vertical transmission was observed in the piglets sampled one day prior to weaning in this study, even in the offspring of positive lactating dams. The lack of detection may have occurred due to an absence of colonization of the piglets at the age they were sampled or due to the fact that laryngeal swabs might not be the most sensitive tool to detect *M. hyopneumoniae* in suckling piglets. It can be hypothesized that colonization starts in the nasal cavity, it is possible that nasal swabs are more sensitive than laryngeal swabs in natural infections in young piglets

5. Conclusions

Under the conditions of this investigation, *M. hyopneumoniae* was detected in self-replacement gilts originated from positive farms, although subpopulations of negative gilts could be detected. The use of various diagnostic assays could potentially increase detection of exposed gilts, however, vaccination practices complicate diagnostic interpretation. The use of molecular typing allowed the identification of *M. hyopneumoniae* variability within and among farms, but also allowed the identification of similar variants at different farms and over time in the same geographical region. These results suggest that bacterial variants should be taken into account when considering acclimation practices, which should be farm-specific.

Competing interests

The authors declare that they have no competing interest.

Acknowledgments

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References

Calsamiglia, M., Pijoan, C., 2000. Colonization state and colostral immunity to

Mycoplasma hyopneumoniae of different parity sows. Vet. Rec. 146, 530–532.
Calsamiglia, M., Pijoan, C., Bosch, G., 1999. Profiling *Mycoplasma hyopneumoniae* in farms using serology and a nested PCR technique. J. Swine Health Prod. 7, 263–268.
Clark, L.K., Armstrong, C.H., Freeman, J.M., Scheidt, A.B., Knox, K., 1991. Investigating the transmission of *Mycoplasma hyopneumoniae* in a swine herd with enzootic pneumonia. Vet. Med. 86, 543–550.
De Castro, L.A., Rodrigues Pedroso, T., Kuchiishi, S.S., Ramenzoni, M., Kich, J.D., Zaha, A., Henning Vainstein, M., Bunselmeyer Ferreira, H., 2006. Variable number of tandem amino acid repeats in adhesion-related CDS products in *Mycoplasma hyopneumoniae* strains. Vet. Microbiol. 116, 275–280.
Dee, S., 1996. The porcine respiratory disease complex: are subpopulations important? Swine Health Prod. 4, 147–149.
Dos Santos, L.F., Sreevatsan, S., Torremorell, M., Moreira, M.A.S., Sibila, M., Pieters, M., 2015. Genotype distribution of *Mycoplasma hyopneumoniae* in swine herds from different geographical regions. Vet. Microbiol. 175, 374–381.
Fano, E., Pijoan, C., Dee, S., 2005. Dynamics and persistence of *Mycoplasma hyopneumoniae* infection on pigs. Can. J. Vet. Res. 69, 223–228.
Fano, E., Pijoan, C., Dee, S., Torremorell, M., 2006. Assessment of the effect of sow parity on the prevalence of *Mycoplasma hyopneumoniae* in piglets at weaning. In: Copenhagen, Denmark,. Proceedings of the IPVS Congress 1. pp. 96.
Fano, E., Pijoan, C., Dee, S., Deen, J., 2007. Effect of *Mycoplasma hyopneumoniae* colonization at weaning on disease severity in growing pigs. Can. J. Vet. Res. 71, 195–200.
Fraile, L., Alegre, A., López-Jiménez, R., Nofrarías, M., Segalés, J., 2010. Risk factors associated with pleuritis and cranio-ventral pulmonary consolidation in slaughter-aged pigs. Vet. J. 184, 326–333.
Furtado, C.S.D., Kummer, R., Wentz, I., Bortolozzo, F.P., 2006. Origem das leitoas de reposição. In: Bortolozzo, F.P., Wentz, I. (Eds.), Suinocultura em Ação. Pallotti, Porto Alegre, pp. 23–33.
Giacomini, E., Ferrari, N., Pitozzi, A., Remistani, M., Giardiello, D., Maes, D., Alborali, G.L., 2016. Dynamics of *Mycoplasma hyopneumoniae* seroconversion and infection in pigs in the three main production systems. Vet. Res. Commun. 40, 81–88.
Maes, D., Deluyker, H., Verdonck, M., Castryck, F., Miry, C., Vrijens, B., Verbeke, W., Viaene, J., de Kruif, A., 1999. Effect of vaccination against *Mycoplasma hyopneumoniae* in pig herds with all-in/all-out production system. Vaccine 17, 1024–1034.
Maes, D., Segales, J., Meyns, T., Sibila, M., Pieters, M., Haesebrouck, F., 2008. Control of *M. hyopneumoniae* infections in pigs. Vet. Microbiol. 126, 297–309.
Meyns, T., Maes, D., Dewulf, J., Vicca, J., Haesebrouck, F., De Kruif, A., 2004. Quantification of the spread of *Mycoplasma hyopneumoniae* in nursery pigs using transmission experiments. Prev. Vet. Med. 66, 264–275.
Michiels, A., Vranckx, K., Piepers, S., Sacristán, R.D.P., Arsenakis, I., Boyen, F., Haesebrouck, F., Maes, D., 2017. Impact of diversity of *Mycoplasma hyopneumoniae* strains on lung lesions in slaughter pigs. Vet. Res. 48, 1–14.
Morris, C.R., Gardner, I.A., Hietala, S.K., Carpenter, T.E., Anderson, R.J., Parker, K.M., 1995. Seroepidemiology study of natural transmission of *Mycoplasma hyopneumoniae* in a swine herd. Prev. Vet. Med. 21, 323–337.
Nathues, H., grosse Beilage, E., Kreienbrock, L., Rosengarten, R., Spergser, J., 2011. RAPD and VNTR analyses demonstrate genotypic heterogeneity of *Mycoplasma hyopneumoniae* isolates from pigs housed in a region with high pig density. Vet. Microbiol. 152, 338–345.
Nathues, H., Woeste, H., Doehring, S., Fahrion, A.S., Doherr, M.G., grosse Beilage, E., 2013. Herd specific risk factors for *Mycoplasma hyopneumoniae* infections in suckling pigs at the age of weaning. Acta Vet. Scand. 55, 1–13.
Pantoja, L.G., Pettit, K., Dos Santos, L.F., Tubbs, R., Pieters, M., 2016. *Mycoplasma hyopneumoniae* variability within a swine operation. J. Vet. Diagn. Invest. 28, 175–179.
Pieters, M., Fano, E., 2016. *Mycoplasma hyopneumoniae* management in gilts. Vet. Rec. 178, 122–123.
Pieters, M., Cline, G.S., Payne, B.J., Prado, C., Ertl, J.R., Rendahl, A.K., 2014. Intra-farm risk factors for *Mycoplasma hyopneumoniae* colonization at weaning age. Vet. Microbiol. 172, 575–580.
Pieters, M., Daniels, J., Rovira, A., 2017. Comparison of sample types and diagnostic methods for *in vivo* detection of *Mycoplasma hyopneumoniae* during early stages of infection. Vet. Microbiol. 203, 103–109.
Pijoan, C., 2003. Prevalence of *Mycoplasma hyopneumoniae* in different parity sows as a preliminary tool for eradication. Res. Rep.: Swine Health 2, 1–5.
R Core Team, 2015. R: A Language and Environment for Statistical Computing [Computer Software]. R Foundation for Statistical Computing, Vienna, Austria Available from. http://www.r-project.org/.
Ramirez, A., Karriker, L.A., 2012. Herd evaluation. In: Zimmerman, J.J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G.W. (Eds.), Diseases of Swine, tenth ed. Wiley-Blackwell, Ames, pp. 15–17.
Roos, L.R., Fano, E., Homwong, N., Payne, B., Pieters, M., 2016. A model to investigate the optimal seeder-to-naïve ratio for successful natural *Mycoplasma hyopneumoniae* gilt exposure prior to entering the breeding herd. Vet. Microbiol. 184, 51–58.
Sibila, M., Calsamiglia, M., Vidal, D., Badiella, L., Aldaz, A., Jensen, J.C., 2004. Dynamics of *Mycoplasma hyopneumoniae* infection in 12 farms with different production systems. Can. J. Vet. Res. 68, 12–18.
Sibila, M., Nofrarias, M., López-Soria, S., Segalés, J., Riera, P., Llopart, D., Calsamiglia, M., 2007. Exploratory field study on *Mycoplasma hyopneumoniae* infection in suckling pigs. Vet. Microbiol. 121, 352–356.
Sibila, M., Pieters, M., Molitor, T., Maes, D., Haesebrouck, F., Segalés, J., 2009. Current perspectives on the diagnosis and epidemiology of *Mycoplasma hyopneumoniae* infection. Vet. J. 181, 221–231.
Takeuti, K.L., Barcellos, D.E.S.N., Lara, A.C., Kunrath, C.F., Pieters, M., 2017. Detection of *Mycoplasma hyopneumoniae* in naturally infected gilts over time. Vet. Microbiol. 203, 215–220.
Thacker, E.L., Minion, F.C., 2012. Mycoplasmosis. In: Zimmermann, J.J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G.W. (Eds.), Diseases of Swine, tenth ed. Wiley-Blackwell, Ames, pp. 779–797.
Thacker, E., 2006. Mycoplasmal diseases. In: Straw, B., Zimmerman, J., DAllaire, S., Taylor, D. (Eds.), Diseases of Swine. Ninth ed. Blackwell Publishing, Ames, pp. 701–717.
Villarreal, I., Vranckx, K., Dutchateaul, L., Pasmans, F., Haesebrouck, F., Jensen, J.C., Nanjiani, I.A., Maes, D., 2010. Early *Mycoplasma hyopneumoniae* infections in European suckling pigs in herds with respiratory problems: detection rate and risk factors. Vet. Med. 55, 318–324.
Vranckx, K., Maes, D., Calus, D., Villarreal, I., Pasmans, F., Hausebrouck, F., 2011. Multiple-locus variable-number tandem-repeat analysis is a suitable tool for differentiation of *Mycoplasma hyopneumoniae* strains without cultivation. J. Clin. Microbiol. 49, 2020–2023.

5. CAPÍTULO IV – TERCEIRO ARTIGO CIENTÍFICO

Artigo a ser submetido na revista Veterinary Microbiology
(de acordo com as normas da revista)

Gilt flow and acclimation as drivers of *Mycoplasma hyopneumoniae* sow herd stability

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Abstract

The objective of this study was to characterize *Mycoplasma* (*M.*) *hyopneumoniae* colonization and seroconversion patterns in negative gilts recently introduced in three positive farms with different types of flows of replacement gilts. Two farms had a continuous flow where negative gilts were introduced at 45 and 65 days of age (doa) and put into contact with older gilts that were coughing, and the third farm had an all-in all-out flow, where negative gilts were placed at 130 doa with culling sows that were coughing. Two replicates of 35 incoming gilts were selected per farm and followed longitudinally with ELISA and real time PCR for *M. hyopneumoniae* every 60 days four or five times. The last sampling was performed prior to farrowing, and 60 piglets were sampled in Farms B and C one day prior to farrowing to evaluate sow to piglet transmission. Moreover, *M. hyopneumoniae* genetic variability was evaluated in gilts by MLVA. The results suggested that similar detection

patterns of *M. hyopneumoniae* by ELISA and PCR were observed within farms regardless the type of replacement flow. Also, no positive gilts were detected by PCR prior to farrowing, including gilts introduced in the farm at older age (130 doa), and no vertical transmission was detected. Moreover, 30.9% of the gilts was never detected positive by PCR during the study, which may have resulted from a possible acclimation failure. In addition, genetic variability analysis revealed a limited number of variants of *M. hyopneumoniae* in the gilts in three different farms.

Keywords: enzootic pneumonia, laryngeal swabs, pigs, real time PCR, serology.

1. Introduction

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is an important pathogen to pig production with economic impact due to losses by enzootic pneumonia and secondary infections in growing and finishing pigs (Thacker, 2006; Thacker and Minion, 2012). Several control methods can be used to control the disease, such as improvement of housing conditions, management and biosecurity practices, gilt acclimation, vaccination and medication with antibiotics effective against *M. hyopneumoniae* and secondary bacteria (Maes et al., 2008).

It has been demonstrated that the dam is the main source of piglet colonization by *M. hyopneumoniae* (Clark et al., 1991; Pijoan, 2003; Pieters et al., 2014), and the dam infectious status at the lactation period is an important risk factor for high prevalence of colonized piglets at weaning (Pieters et al., 2014). This is relevant, as the colonization of piglets at weaning is an indicator of prevalence and severity of *M. hyopneumoniae* lesions at slaughter (Fano et al., 2007).

The gilts have a major role in the transmission of *M. hyopneumoniae* to their offspring, since a higher proportion shed the bacterium at first farrowing when compared to old parity dams (Calsamiglia and Pijoan, 2000; Fano et al., 2006). Gilt replacement is a common practice in the swine industry, and this should be carried out purchasing gilts from herds with similar of higher sanitary status (Amass and

Baysinger, 2006). However, many *M. hyopneumoniae* endemically infected farms purchase replacement gilts from negative farms and, in this situation, incoming gilts should be acclimated for *M. hyopneumoniae* in order to control the disease (Pieters and Fano, 2016).

A gilt acclimation protocol has been suggested by Roos et al. (2016). It consists on the identification of coughing pigs which tested positive for *M. hyopneumoniae* (Ct value less than 30) by real time PCR. To achieve a successful exposure to *M. hyopneumoniae,* a proportion of six positive pigs (seeders) should be housed with four naïve gilts for a 4-week period in the same pen. Due to the fact that the infection by *M. hyopneumoniae* causes a chronic lung infection and that pigs can shed and potentially transmit the bacterium to susceptible pigs for up to 214 days (Pieters et al., 2009), gilt acclimation should be performed early in life for a proper recovery by the first farrowing (Pieters and Fano, 2016).

To date, little information is available on the dynamics of *M. hyopneumoniae* infection in purchased gilts over time. Moreover, to the best of the authors knowledge, this is the first study to evaluate the effectiveness of acclimation using two types of flows of replacement gilts. Therefore, the objective of this study was to characterize *M. hyopneumoniae* colonization and seroconversion patterns in negative gilts recently introduced into two replacement programs (continuous and all-in all-out flows).

2. Materials and Methods

2.1.Ethics statement

The management with the animals enrolled in this study followed the usual pattern in the farms. The study was conducted under the approval of the Institutional Animal Care and Use Committee of the University of Minnesota.

73 **2.2. Farms**

74 Three sow farms (A, B and C), located in two states in the Midwestern United States, with
75 different gilt flows were chosen for this study. The farms were selected based on the number of sows
76 (greater than 1000 sows), they were positive for *M. hyopneumoniae* with historic of respiratory clinical
77 signs, and the replacement of gilts was performed purchasing negative gilts.

78 Farm A was a 5200-sow farm with a continuous flow management of replacement gilts. In this
79 farm, negative gilts were housed in the gilt development unit (GDU) with approximately 65 days of age
80 (doa). Acclimation for *M. hyopneumoniae* was performed by the direct contact of the negative
81 incoming gilts with selected older gilts with clinical signs of coughing. The presumed positive animals
82 (seeders) were not tested. Historically, seven months prior to the beginning of the study, the farm had a
83 Porcine Reproductive and Respiratory Syndrome (PRRS) outbreak, and it was considered stabilized
84 four months before the beginning of this study. Gilts were vaccinated against Influenza (IAV) and
85 PRRS, and they were not vaccinated against *M. hyopneumoniae*. Treatment with Lincomycin was
86 performed when respiratory clinical signs were apparent.

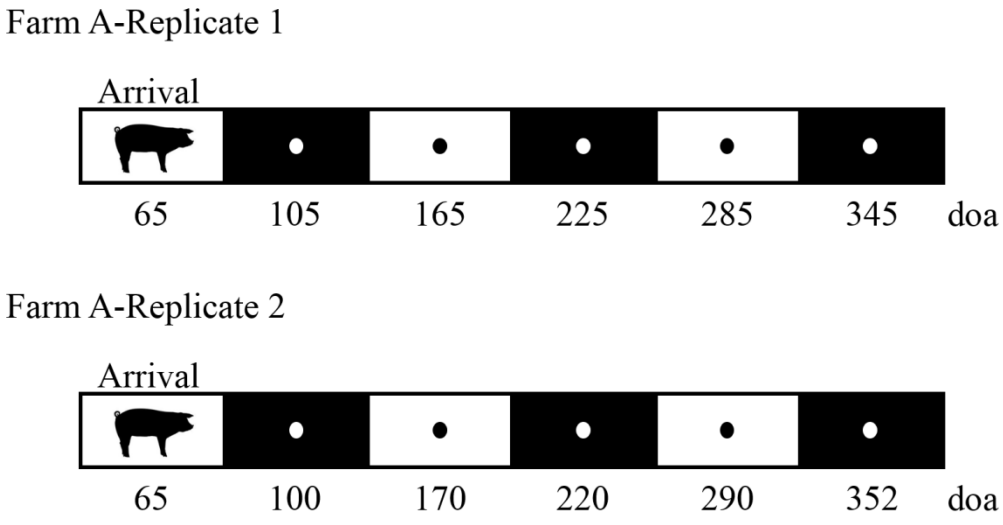
87 Farm B was a 2600-sow farm with an all-in all-out management of replacement gilts. Gilts were
88 allocated in the GDU with approximately 130 doa and the acclimation was performed by the direct
89 contact with culling sows (locomotion problems, double returns and abortions) that were coughing. The
90 presumed seeders were not tested. The farm was endemic and stable for PRRS and gilts were
91 vaccinated against IAV, PRRSv, Circovirus type 2 (PCV2) and *M. hyopneumoniae* (commercial
92 bacterin two and three weeks post placement). Gilts were treated with Tulathromycin if coughing was
93 observed.

94 Farm C was a 3000-sow air filtered farm with a continuous flow management of replacement
95 gilts. Gilts were housed in the GDU with approximately 45 days of age and acclimated for *M.*
96 *hyopneumoniae* by the direct contact with older groups of gilts with clinical signs of coughing. The

presumed seeders were not tested. The farm was endemic for PRRS and gilts were vaccinated against PCV2, ileitis and *M. hyopneumoniae* (commercial bacterin three and eight weeks post placement) and exposed to PRRSv at three and eight weeks after placement in the GDU. Gilts were treated at arrival with Tiamulin and Amoxicillin in the water for five days.

2.3. Experimental design

At each farm, two replicates of 35 replacement gilts were randomly selected after arrival at the GDU, ear tagged and followed longitudinally. Blood samples and laryngeal swabs were collected four or five times every 60 days, approximately, and the last sample was collected prior to farrowing. A graphic representation of the experimental design is shown in Figure 1. One day prior to weaning, ten and two litters (five piglets per litter) were collected in Farm B and C, respectively, using nasal swabs to evaluate vertical transmission by the detection of *M. hyopneumoniae* by real time PCR.



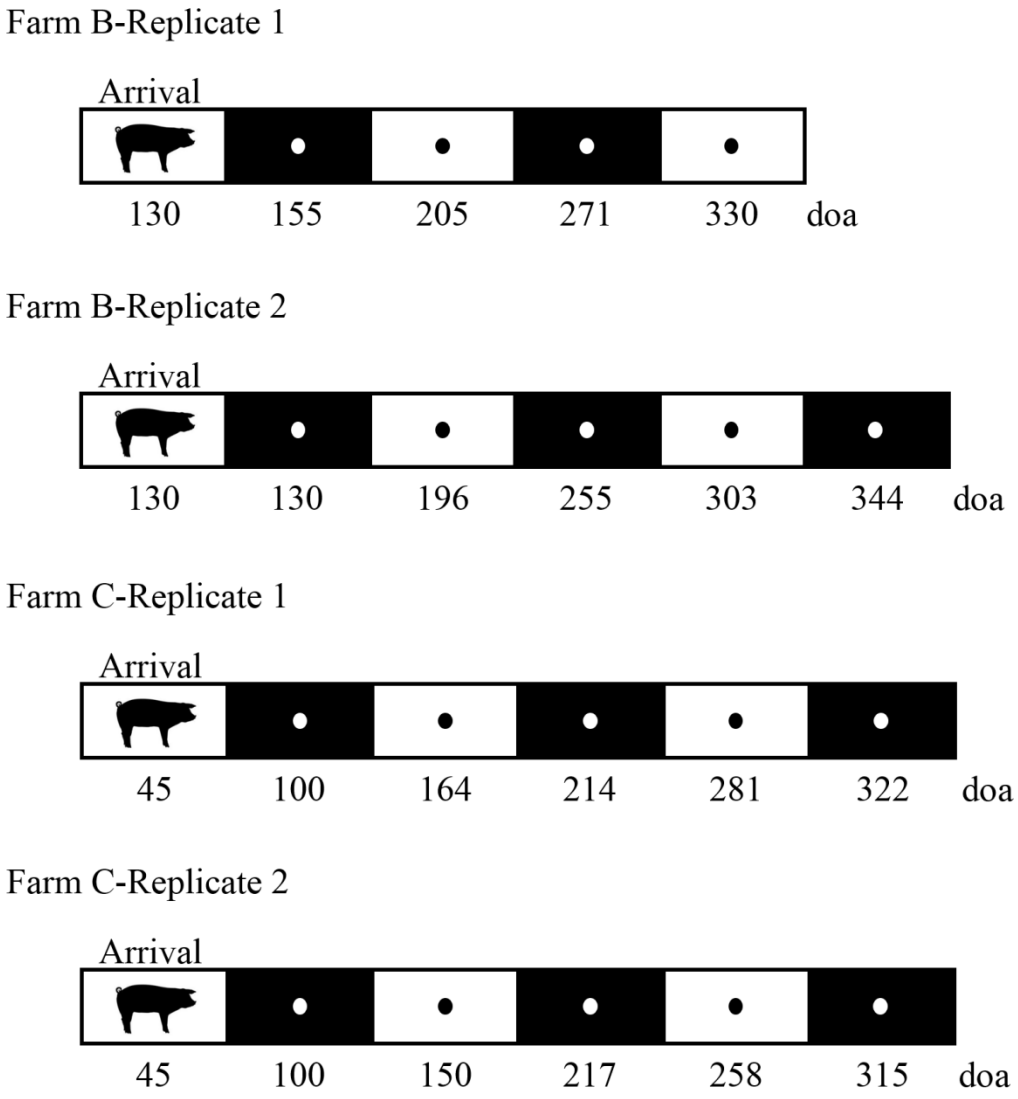


Figure 1. Schematic representation of the study experimental design. Two replicates of 35 replacement gilts were selected and ear tagged at arrival in three farms (A, B, C) and followed longitudinally for four or five samplings. Every 60 days, approximately, blood and laryngeal swabs were collected for ELISA and real time PCR for *M. hyopneumoniae*, respectively. Dots represent each sampling.

2.4. Sampling

Blood samples were collected from the caval vein using vacuum tubes (BD Vacutainer® Blood Collection Tubes, Franklin Lakes, NJ, USA) and sterile needles (BD Vacutainer® Blood Collection Needles, Franklin Lakes, NJ, USA) as described by Ramirez and Karriker (2012).

Laryngeal swabs were collected with the insertion of sterile swabs (BBL™ CultureSwab™, Sparks, MD, USA) in the mouth cavity after they reached the larynx with the aid of a mouth gag and a laryngoscope, as previously described by Pieters et al. (2017). All swabs were refrigerated, delivered to the laboratory and frozen at -20°C until analysis.

2.5. Sample processing and testing

Blood samples were processed for serum separation using a centrifuge (Allegra X-14, Beckman Coulter, Inc., Indianapolis, IN, USA) at 1500 x g x 10 min at 4ºC. Serum was aliquoted and stored at -20°C until analysis. The antibodies detection for *M. hyopneumoniae* was performed using a commercial ELISA test (Idexx Laboratories, Westbrook, ME, USA), following manufacturer's instructions. Samples were considered positive when S/P ratio ≥ 0.4.

Laryngeal swabs were processed and DNA extraction was performed with MagMAX™-96 Viral RNA isolation kit and MagMAX™ Express-96 Magnetic Particle Processor (Life Technologies, Grand Island, NY, USA). The real time PCR was performed with VetMAX™ qPCR Master mix and VetMAX™ *M. hyopneumoniae* reagents kit (Life Technologies, Grand Island, NY, USA), according to manufacturer's protocol. Two positive and two negative controls were used in each plate, and samples were tested individually in duplicate. Samples were considered positive for real time PCR when Ct value was lower than 37.

A total of 14 samples (7 from Farm A, 4 from Farm B, and 3 from Farm C) with Ct values lower than 31 were characterized using Multiple Locus Variable-number tandem repeats Analysis (MLVA)

typing, as previously described by Dos Santos et al. (2015). Analysis was performed in Bionumerics version 7.0 (Applied Maths, East Flanders, Belgium).

2.6. Statistical analysis

The number of laryngeal swabs or serum detected positive for *M. hyopneumoniae* by PCR or ELISA, respectively, was used to calculate the prevalence in each farm and sampling. Multiple comparison tests with the Bonferroni adjustment were performed in R v3.2 (R Core Team, 2015) to compare prevalence among samplings with a confidence interval of 95%. Statistical significance was considered when p-values were less than 0.05. On each sampling event, the confidence interval was calculated in R v3.2 (R Core Team, 2015) and Agresti-Coull interval was calculated for prevalences equal to 0%.

3. Results

Mycoplasma hyopneumoniae antibodies detection by ELISA

The prevalence of positive gilts for the detection of circulating antibodies for *M. hyopneumoniae* is shown in Figures 2 to 4. The three farms showed low prevalence of positive gilts in the first sampling in both replicates, except replicate 2 from Farm C. A progressive increase ($p < 0.05$) on prevalence was observed from the first to the third sampling in Farm A and B, and a decrease ($p < 0.05$) was detected in both replicates from Farm A, and in the second replicate from Farm B. The prevalence of positive gilts by ELISA remained high up to the last sampling in Farm C. The incidence from the second to the fifth sampling is demonstrated in Figure 5.

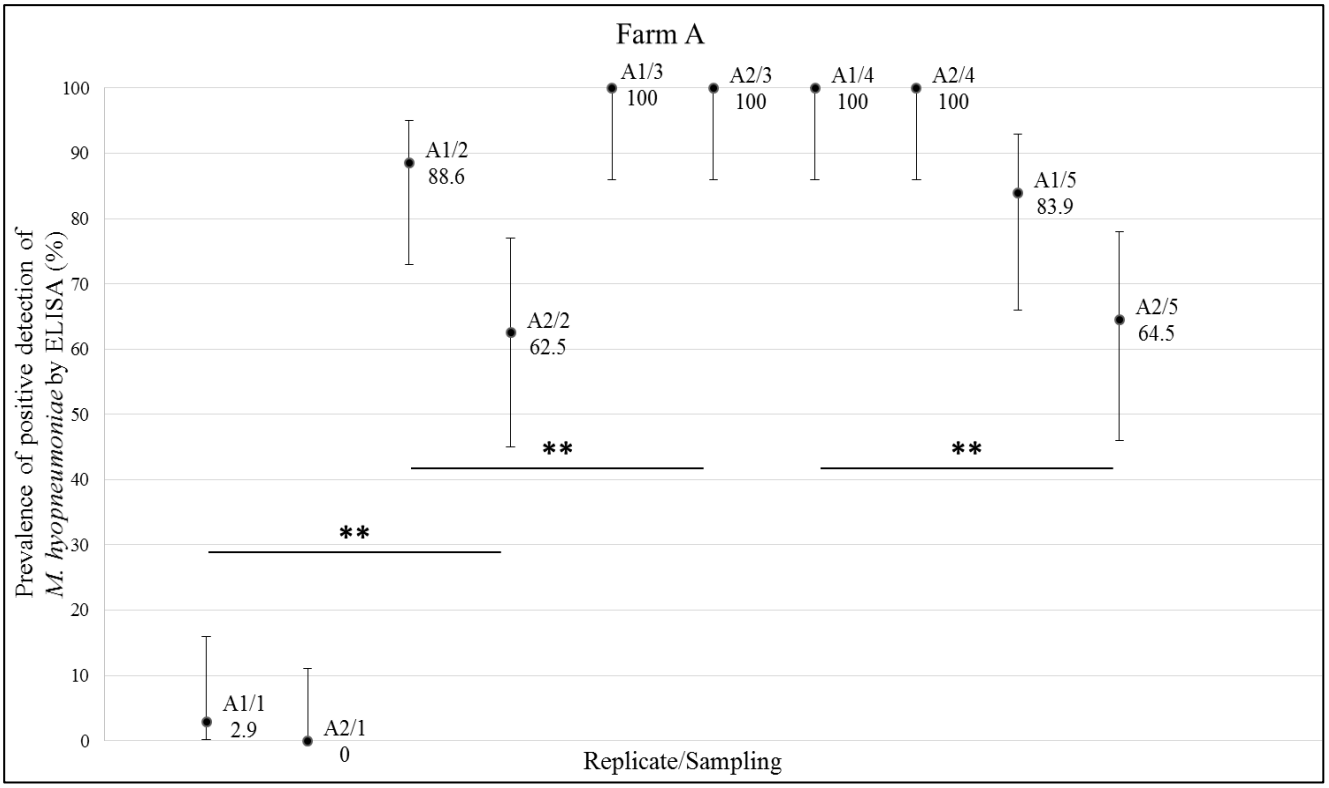


Figure 2. Prevalence of *M. hyopneumoniae* positive gilts over time by ELISA in Farm A. The letter corresponds to the farm (A), the first number indicate the replicate (1 or 2), and the second number corresponds to the sampling event (1 to 5). Double asterisks represent significant difference ($p < 0.05$) among samplings in replicates 1 and 2, based on multiple comparisons test and Bonferroni adjustment.

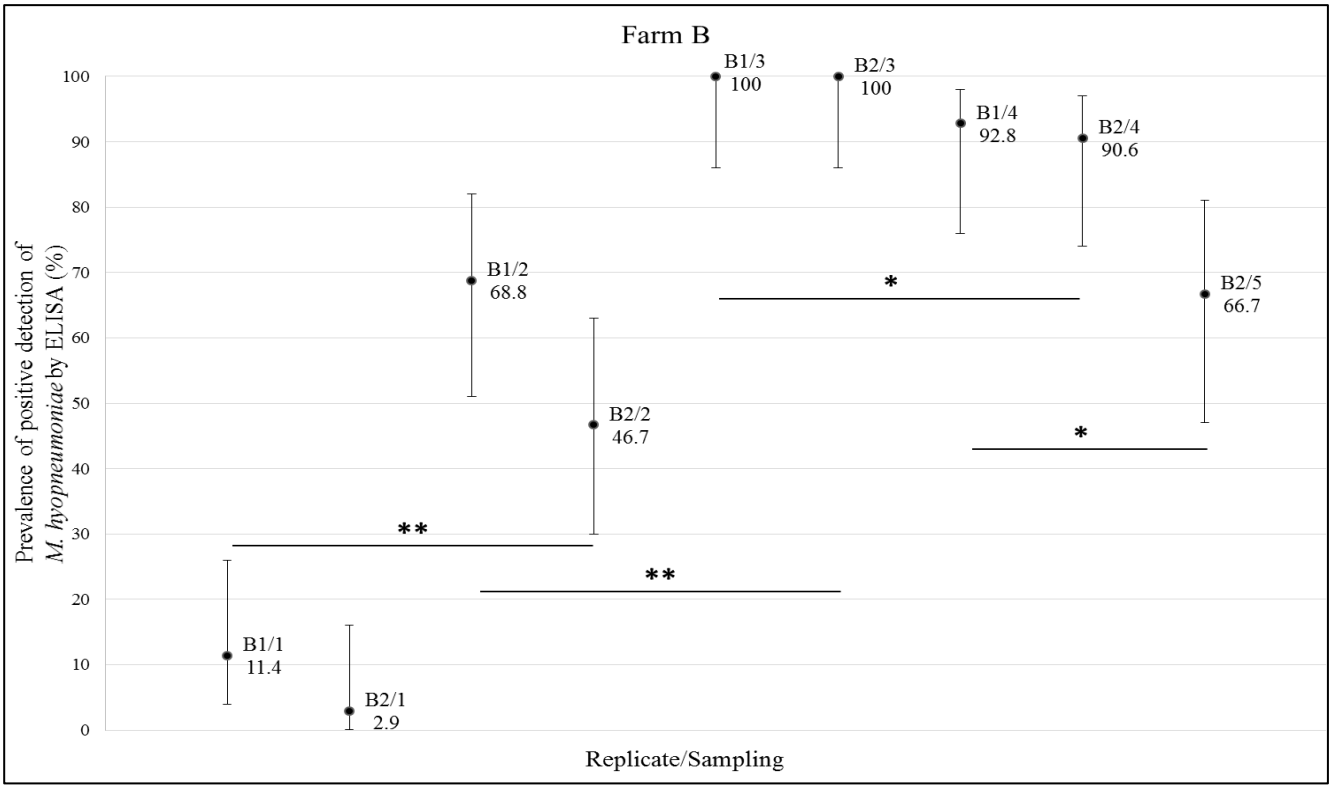


Figure 3. *Mycoplasma hyopneumoniae* detection in gilts over time after ELISA testing in Farm B. The letter corresponds to the farm (B), the first number indicate the replicate (1 or 2), and the second number corresponds to the sampling event (1 to 5). Single asterisks represent significant difference ($p < 0.05$) among samplings in replicate 2, and double asterisks indicate statistical difference ($p < 0.05$) in replicates 1 and 2, based on multiple comparisons test and Bonferroni adjustment.

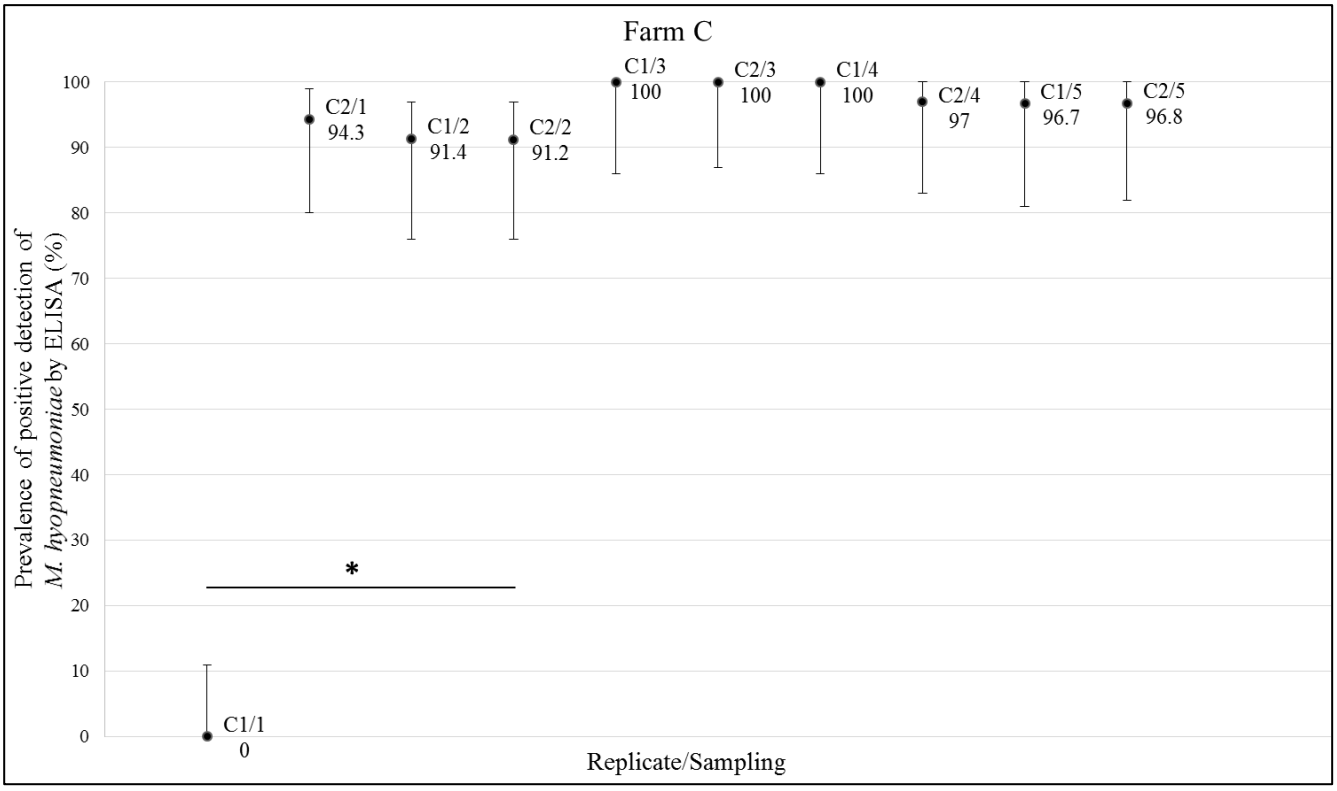


Figure 4. Positive detection of circulating antibodies against *M. hyopneumoniae* over time in Farm C. The letter corresponds to the farm (C), the first number indicate the replicate (1 or 2), and the second number corresponds to the sampling event (1 to 5). Single asterisk represents significant difference ($p < 0.05$) among samplings in replicate 1, based on multiple comparisons test and Bonferroni adjustment.

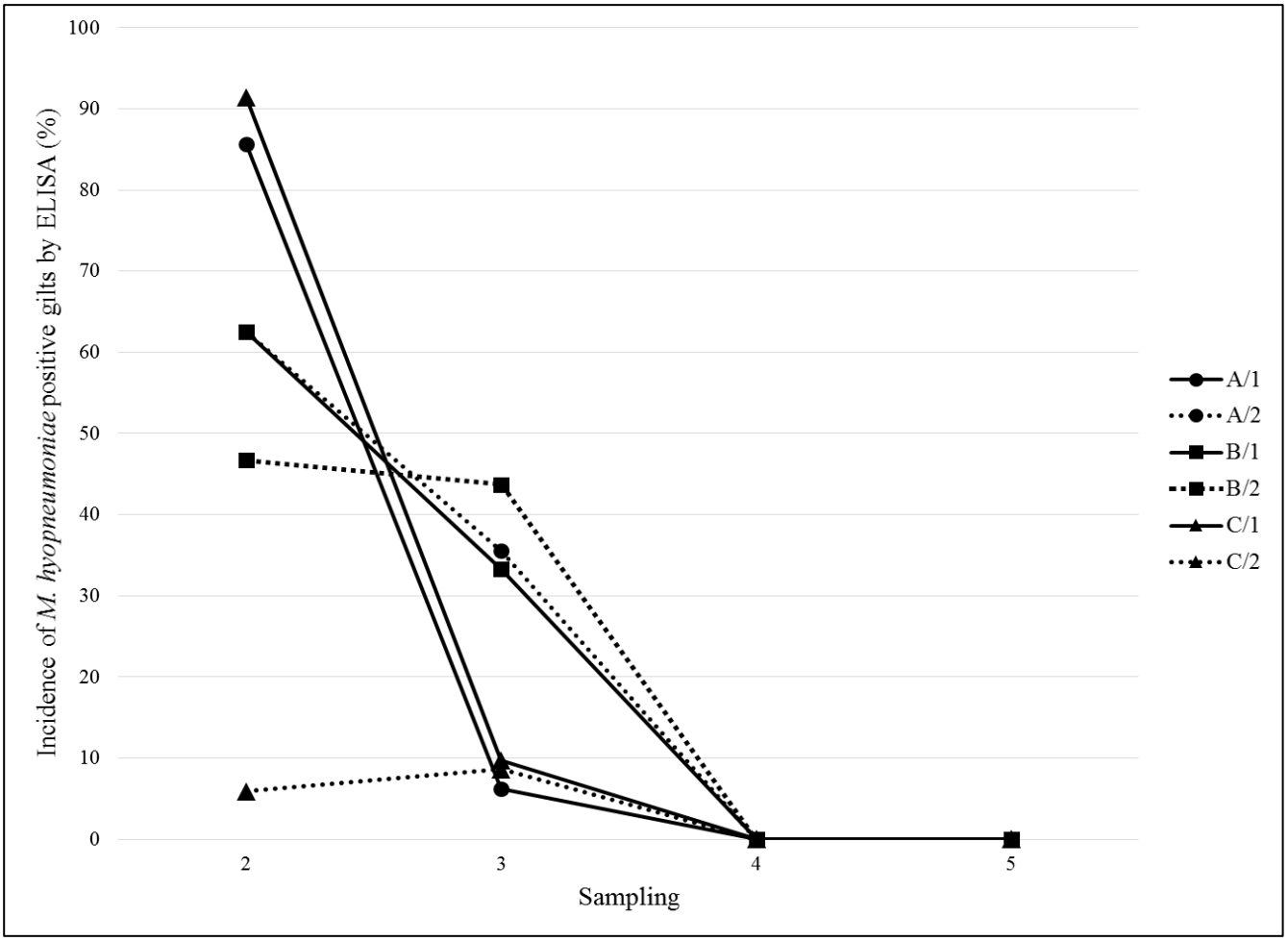


Figure 5. Incidence of positive gilts for *M. hyopneumoniae* by ELISA in samplings two to five in Farms A, B and C. Dots represent incidences in Farm A, squares correspond to Farm B, and triangles represent Farm C. Solid and dash lines represent the replicates (1 and 2, respectively).

Mycoplasma hyopneumoniae detection by real time PCR

In the first sampling, none of the gilts of the study was detected positive for *M. hyopneumoniae* by real time PCR. A significant increase ($p < 0.05$) on prevalence of positive gilts was detected from the first to the second sampling in both replicates in all farms (Figures 6 to 8). In addition, significant decreases on the prevalence of positive gilts were detected from the second to the fourth sampling in all farms in at least one replicate, and no detection was observed in the fourth and fifth samplings in the three farms in both replicates. The incidence in each sampling time is demonstrated in Figure 9.

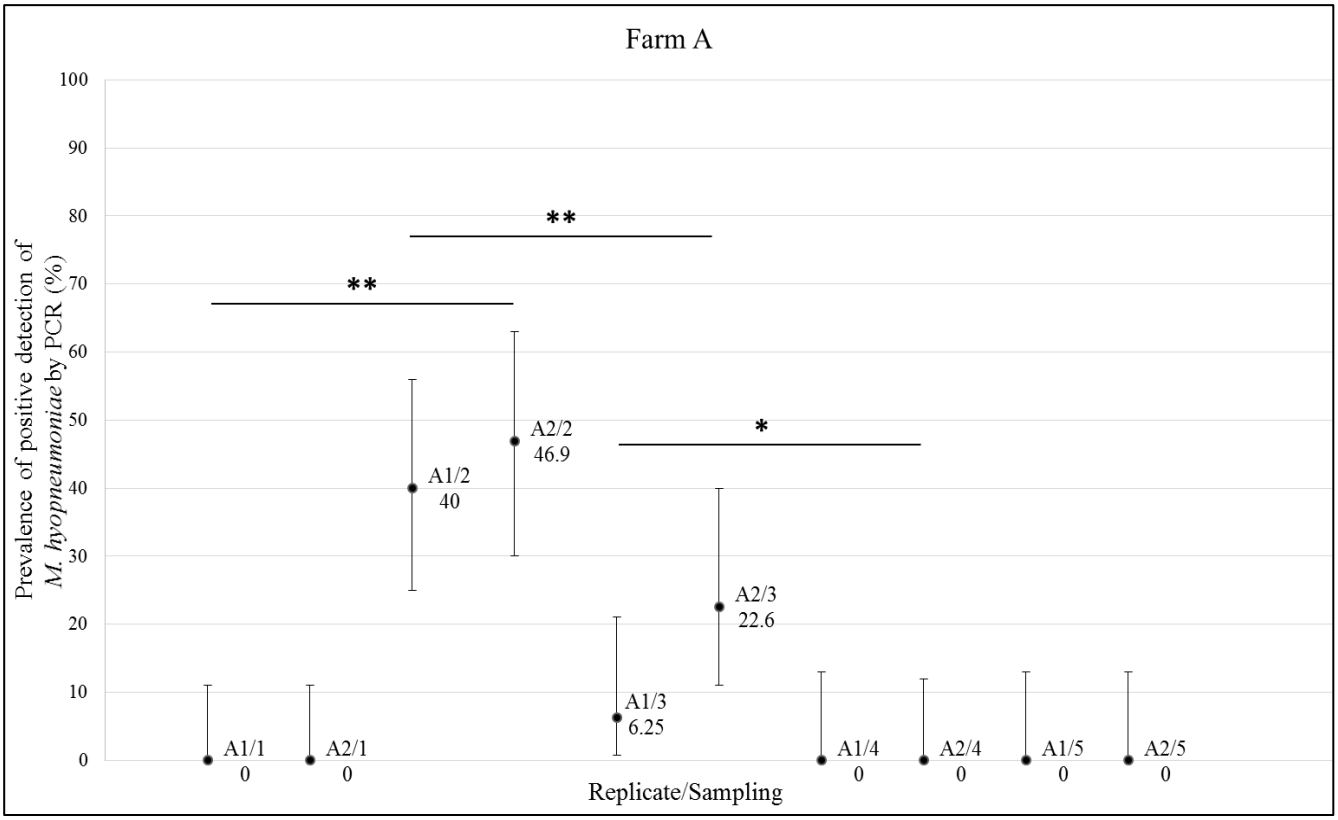


Figure 6. *Mycoplasma hyopneumoniae* prevalence by PCR over time in Farm A. The letter corresponds to the farm (A), the first number indicate the replicate (1 or 2), and the second number corresponds to the sampling event (1 to 5). Double asterisks indicate statistical difference ($p < 0.05$) among samplings in replicates 1 and 2. Single asterisk represents significant difference ($p < 0.05$) between samplings in replicate 2, based on multiple comparisons test and Bonferroni adjustment.

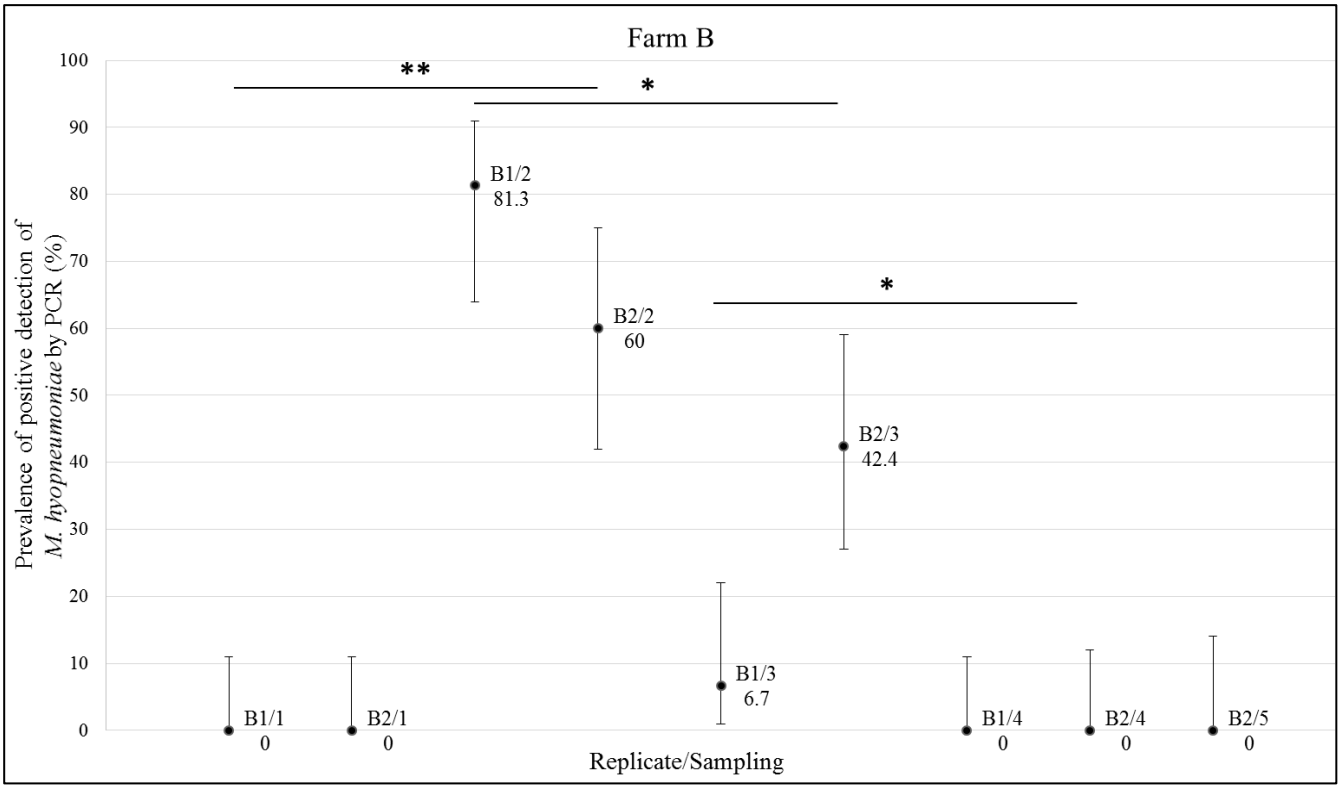


Figure 7. Detection of *M. hyopneumoniae* in gilts over time after PCR analysis. The letter corresponds to the farm (B), the first number indicate the replicate (1 or 2), and the second number corresponds to the sampling event (1 to 5). Double asterisk indicates statistical difference ($p < 0.05$) between samplings in replicates 1 and 2. Single asterisks represent significant difference ($p < 0.05$) among samplings in replicate 1 or 2, based on multiple comparisons test and Bonferroni adjustment.

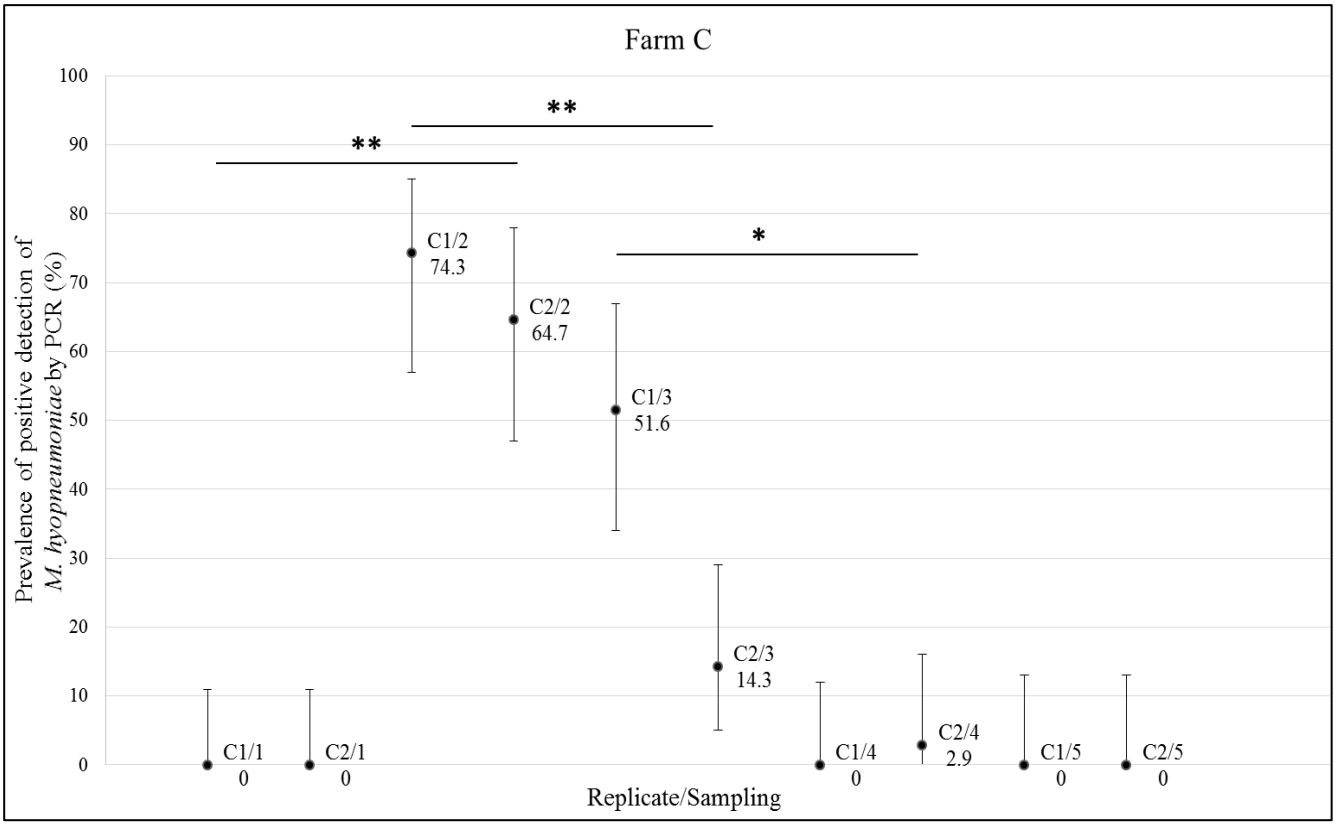


Figure 8. Prevalence of *M. hyopneumoniae* positive gilts by PCR in a longitudinal study. The letter corresponds to the farm (C), the first number indicate the replicate (1 or 2), and the second number corresponds to the sampling event (1 to 5). Double asterisks indicate statistical difference ($p < 0.05$) among samplings in replicates 1 and 2. Single asterisk represents significant difference ($p < 0.05$) between samplings in replicate 1, based on multiple comparisons test and Bonferroni adjustment.

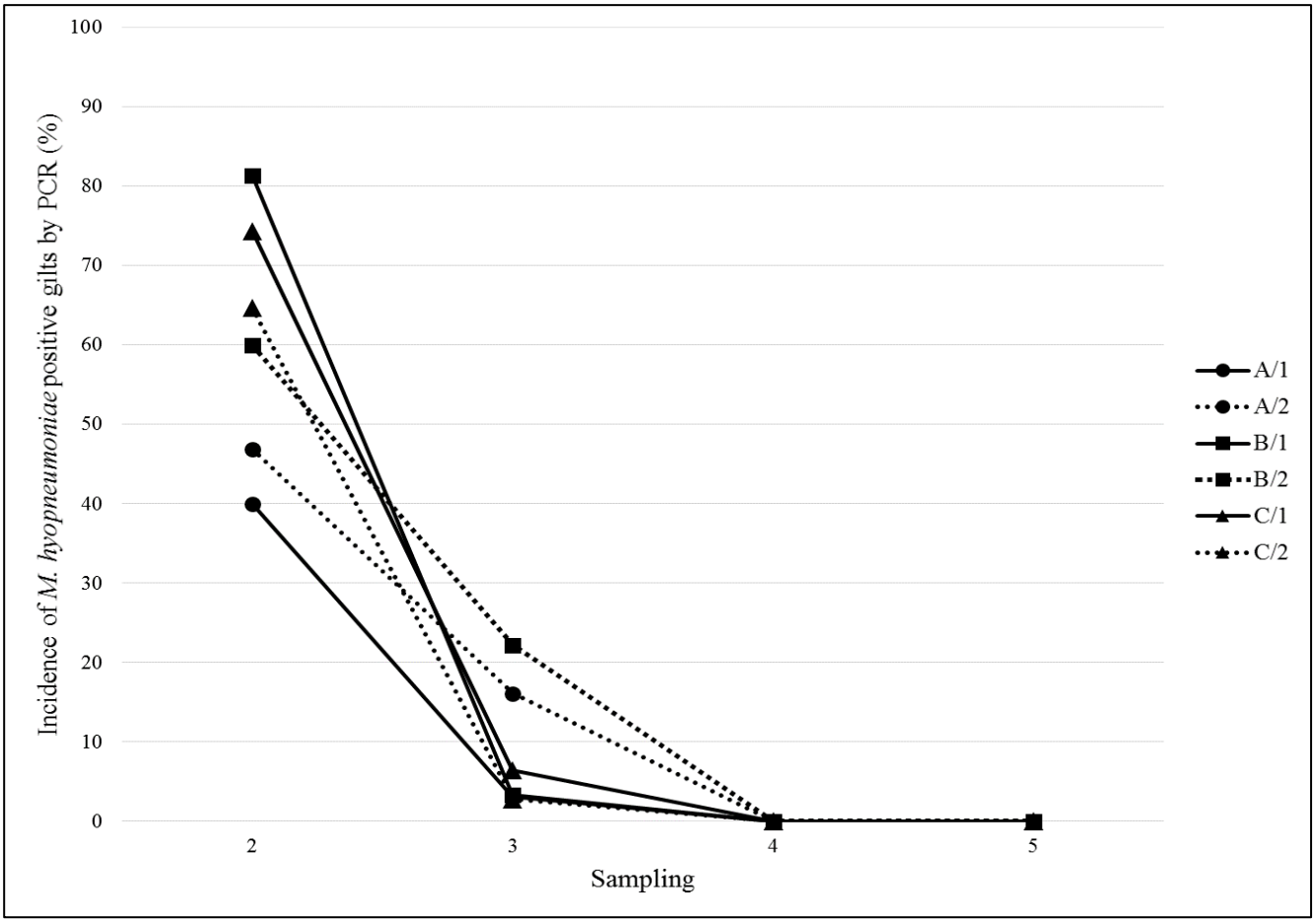


Figure 9. Incidence of positive gilts for *M. hyopneumoniae* by PCR in samplings two to five in Farms A, B and C. Dots represent incidences in Farm A, squares correspond to Farm B, and triangles represent Farm C. Solid and dash lines represent the replicates (1 and 2, respectively).

The detection pattern of *M. hyopneumoniae* by real time PCR was analyzed individually in 165 gilts (60 in Farm A, 46 in Farm B, and 59 in Farm C) that remained in the herds until the completion of the study. The results indicated similar patterns among farms (Table 1). A single detection in the second or in the third sampling corresponded to 53.9% of all patterns observed over time. The lack of detection in all sampling times was responsible for 30.9% of the gilts, and the number of positive gilts in the second and in the third samplings represented 15.2%.

Table 1. Detection pattern of *M. hyopneumoniae* by real time PCR in acclimated purchased gilts over time in three farms.

Farm	***M. hyopneumoniae* detection pattern by PCR**					**Prevalence %**
	First Sample	**Second Sample**	**Third Sample**	**Fourth Sample**	**Fifth Sample**	**(positive/tested)**
A	-	-	-	-	-	45 (27/60)
	-	+	-	-	-	40 (24/60)
	-	-	+	-	-	10 (6/60)
	-	+	+	-	-	5 (3/60)
B	-	+	-	-	-	54.3 (25/46)
	-	-	-	-	-	23.9 (11/46)
	-	+	+	-	-	10.9 (5/46)
	-	-	+	-	-	10.9 (5/46)
C	-	+	-	-	-	44.1 (26/59)
	-	+	+	-	-	28.8 (17/59)
	-	-	-	-	-	22 (13/59)
	-	-	+	-	-	5.1 (3/59)

A total of 60 piglets were tested one day prior to weaning in Farm B and C and all of them resulted negative for *M. hyopneumoniae* by real time PCR (data not shown).

Multiple Locus Variable-number tandem repeats Analysis (MLVA)

The MLVA typing of 14 samples revealed the presence of five different variants of *M. hyopneumoniae* within farms (Figure 10). Two variants (15-21 and 13-21, which was the most prevalent) were detected in Farm A, two variants (3-13 and 3-14) in Farm B, and one in Farm C (13-19).

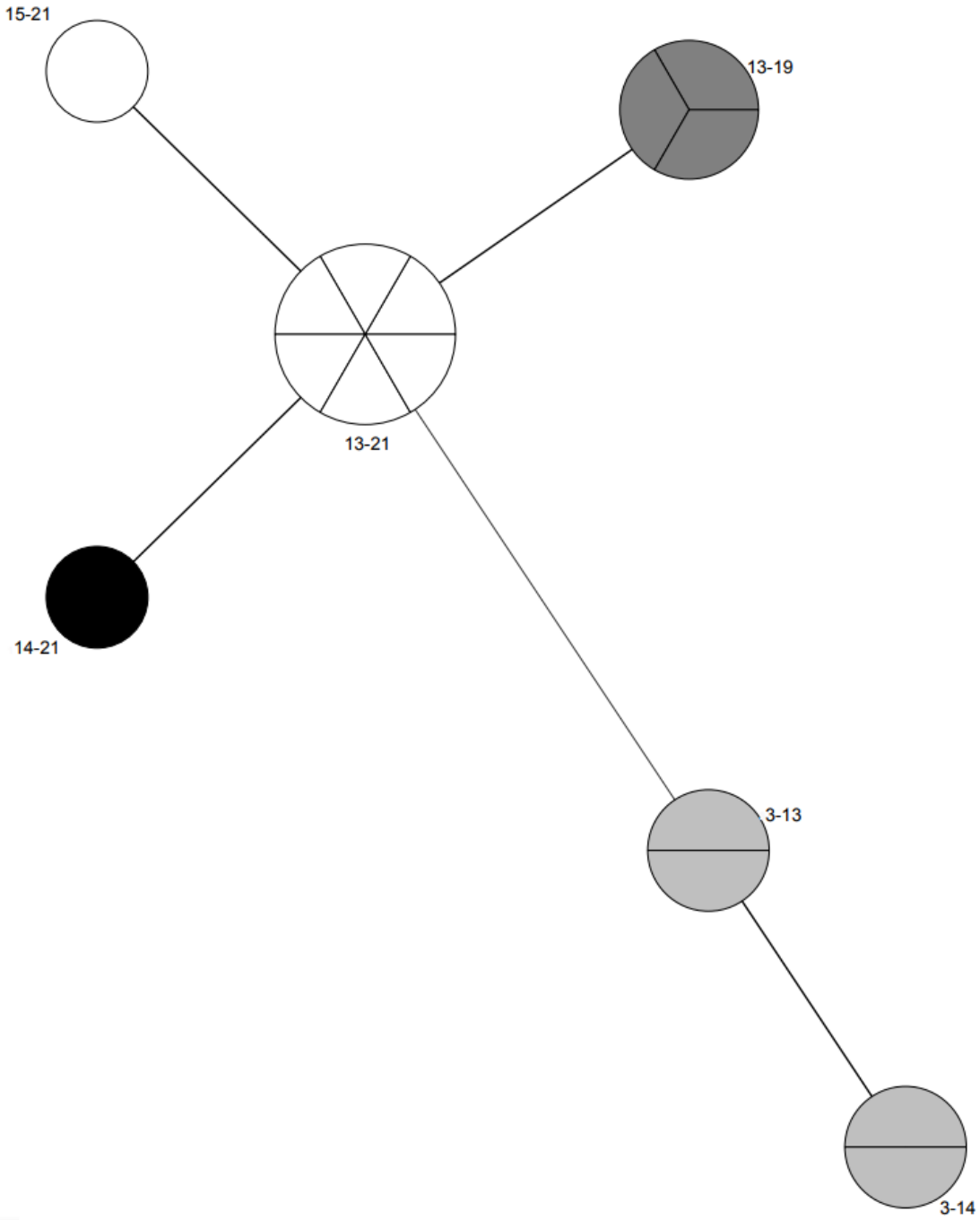


Figure 10. Minimum spanning tree of *M. hyopneumoniae* samples characterized by MLVA. Farm A is represented by white nodes, light grey nodes represent Farm B, and Farm C is represented by dark grey node. The U.S. reference strain is represented by the black node. A total of five variants were detected within farms (two in Farm A and Farm B, and one in Farm C).

4. Discussion

A longitudinal study was performed to evaluate the infection dynamics of *M. hyopneumoniae* in negative gilts introduced in positive farms after two different acclimation protocols were conducted (all-in all-out - two farms or continuous flow - one farm). Antibody detection (ELISA) and real time PCR were used in order to evaluate the detection dynamics over time. Moreover, genetic characterization within farms was evaluated by MLVA testing.

Antibodies detection by ELISA showed an increase in prevalence ($p < 0.05$) in the second sampling in all farms and replicates, except for the second replicate from Farm C. Even though a decrease ($p < 0.05$) was detected in both replicates from Farm A and in the second replicate from Farm B, the prevalence remained high (above 64%) from the third to the last sampling in all farms. Since gilts were not vaccinated against *M. hyopneumoniae* in Farm A, detection of positive gilts indicates seroconversion after natural exposure. Only 2.9% of them was positive in the first sampling (35 days after housing in the GDU), and 100% of the gilts was positive in the third sampling (155 and 160 days after their introduction in the GDU). These results suggest that seroconversion for *M. hyopneumoniae* takes a long time to occur, however, antibodies remain detectable for a long period, as already reported by Morris et al. (1995). In Farm B, gilts were vaccinated against *M. hyopneumoniae* 10 days before the first sampling in gilts from replicate 1, and two weeks after the first sampling in gilts from replicate 2. Despite the fact that seroconversion occurs 22 days post infection/vaccination, on average (Sorensen et al., 1997), it is possible that gilts from replicate 1 had seroconverted earlier. It is already known that

seroconversion depends on *M. hyopneumoniae* strain. Pigs infected with highly virulent strains have shown a faster seroconversion than animals infected with low pathogenicity strains (Meyns et al., 2004; Vicca et al., 2003). However, one gilt (2.9%) was detected positive before vaccination against *M. hyopneumoniae*. Since the blood was collected in the same day the gilts were housed in the GDU, this gilt had no time to seroconvert after natural exposure. In this case, it is hypothesized that the positive gilt could represent a false positive in the ELISA test, as cross reaction may occur with *M. flocculare* (Calsamiglia et al., 1999). The gilts from Farm C were vaccinated for the first time 34 days prior to the first sampling and 94.3% of the gilts from replicate 2 was detected positive. However, no gilts in replicate 1 were detected positive for *M. hyopneumoniae* by ELISA at this moment. The lack of detection may indicate a vaccination or acclimation failures, since the gilts would have enough time to seroconvert after immunization or natural exposure.

The incidence of positive gilts for *M. hyopneumoniae* by ELISA showed that highest incidence was detected in the second sampling, which decreased over time. Moreover, it was not longer possible to detect new cases in the fourth and fifth samplings. It is important to note that Farm C had the highest (replicate 1) and the lowest incidence (replicate 2) in the second sampling, which could be explained by the fact that no positive gilts were detected in the first sampling in replicate 1 and 94.3% of the gilts from replicate 2 was positive for *M. hyopneumoniae* in the first sampling.

The real time PCR results showed a lack of positive gilts in the first sampling in all farms, and a significant increase ($p < 0.05$) in the second sampling (150-205 doa), when prevalence ranged from 40 to 81.3% among farms. Similar results were observed in non-acclimated self-replacement gilts in three multiplier farms in a longitudinal study, where 47-67.4% of the gilts was detected positive for *M. hyopneumoniae* by PCR when they were 150 doa (Takeuti et al., 2017b). In our study, a significant decrease ($p < 0.05$) on prevalence of positive gilts was observed in the subsequent samplings and all gilts were detected negative for *M. hyopneumoniae* prior to farrowing, regardless the type of

284 replacement flow, suggesting that the gilts may not shed *M. hyopneumoniae* at this moment and may
285 not colonize the suckling piglets. Infection did not occur subsequently, as all piglets were negative one
286 day prior to weaning. On the other hand, Takeuti et al. (2017b) observed up to 15.7% positive non-
287 acclimated self-replacement gilts at farrowing or lactation (330 doa). The results of these two studies
288 indicate that similar detection of *M. hyopneumoniae* was observed in gilts in young age, however, non-
289 acclimated self-replacement gilts are potentially more likely to shed *M. hyopneumoniae* at farrowing or
290 lactation than acclimated purchased gilts.

291 Several sites have been used to detect *M. hyopneumoniae* by PCR, however comparative studies
292 have shown that tracheo-bronchial (Kurth et al., 2002; Fablet et al., 2010) and laryngeal swabs (Pieters
293 et al., 2017) are the most sensitive tolls. Under experimental conditions, *M. hyopneumoniae* can be
294 detected in 81% and 100% of the pigs after 5 and 28 days post inoculation, respectively (Pieters et al.,
295 2017). After direct contact of naïve pigs with seeders, *M. hyopneumoniae* was detected in 41% of the
296 animals 14 days after exposure using laryngeal swabs (Roos et al., 2016). Even though the high
297 performance of laryngeal swabs in *M. hyopneumoniae* detection by PCR, no positive gilts were
298 detected up to 55 days after their placement in the GDU. It is hypothesized that a sufficient bacterial
299 load is necessary to be detected in the larynx in natural infections, as already proposed by the study
300 conducted by Takeuti et al. (2017a). In this study, a single detection, in the second or in the third
301 sampling represented 50% of gilts in Farm A, 65.2% in Farm B, and 49.2% in Farm C, which was the
302 most common detection pattern regardless the type of flow. A lack of detection was observed in 45%,
303 23.9% and 22% of the gilts from Farm A, B, and C, respectively. The average of negative gilts among
304 farms was 30.9%, and similar prevalence (30.7%) was observed in self-replacement gilts in a previous
305 study (Takeuti et al., 2017b). It is possible that we failed to detect positive gilts, since the test is not
306 100% sensitive, or an acclimation failure could have occurred as the shedding status of the contact
307 animals (seeders) was not investigated. The acclimation was performed by the contact of incoming

negative gilts with pigs that were coughing, which is not a pathognomonic clinical sign of *M. hyopneumoniae* infection, as other primary or secondary pathogens can produce similar symptoms. The presence of dust or gas, such as ammonia, could have increased the frequency of coughing animals inside the barns, since the study was conducted during the winter where such high gas exposure was present. Hypothetically, the ideal acclimation process should be performed identifying coughing animals, collecting laryngeal swabs for *M. hyopneumoniae* detection by real time PCR, and selecting those animals with low Ct values. In one study, it was suggested that incoming gilts should be housed in the same pens with shedding animals in order to provide a direct contact (nose to nose) for four weeks to guarantee a proper acclimation (Roos et al., 2016). It is important to note that the highest prevalence of negative gilts during the entire study was observed in Farm A, which contributed with 45% of the gilts. It is possible that an incorrect exposure of the incoming gilts with older gilts may have occurred, or it could indicate the presence of a low virulent strain in herd, which may lead to a lower transmission rate of *M. hyopneumoniae* among pigs (Meyns et al., 2004).

The incidence of positive gilts for *M. hyopneumoniae* by PCR showed that the highest incidence, which ranged from 40% to 81.3%, was observed in the second sampling (66 to 119 days after gilts were placed in the GDU). These results indicate that the majority of the gilts became infected by *M. hyopneumoniae* after acclimation. It has been suggested that the gilt acclimation should be performed at approximately 50 doa due to the fact that *M. hyopneumoniae* infection is characterized by a prolonged shedding (Pieters and Fano, 2016). In our study, we compared the detection dynamics of *M. hyopneumoniae* in 45 and 65 doa purchased gilts (continuous flow) with 130 doa gilts (all-in all-out flow) and no differences in the detection patterns were observed. Even though gilts were housed in the GDU when they were 130 doa, up to 81.3% of them was detected positive for *M. hyopneumoniae* in the second sampling (75 days after housing in the GDU). Moreover, none of these gilts shed *M.*

hyopneumoniae prior to farrowing and no vertical transmission was observed, demonstrating that 130 doa acclimated gilts had enough time to heal prior to farrowing.

The genetic variability analysis of *M. hyopneumoniae* in gilts from three farms indicated a limited number of different variants, as previously described by other authors in the U.S. (Pantoja et al., 2016). In Brazil, two studies detected a different situation, indicating a circulation of a higher number of variants. In the study of Takeuti et al. (2017b), high variability was detected in self-replacement gilts by MLVA. Seventeen variants were identified within three herds, and up to 11 variants were detected in one single farm. The same situation was demonstrated by Dos Santos et al. (2015), who observed 39 different MLVA types in clinical specimens. These differences may reflect specific variables differing in Brazilian and North American situations, such as housing, environmental control and number of origins involved in multi-sourcing management. However, it is important to note that a low number of samples were analyzed in our study, which may have resulted in detection of a limited number of variants.

5. Conclusions

The results of this study contributed to understand the effectiveness of different replacement flows (all-in all-out or continuous). The detection dynamics of *M. hyopneumoniae* in acclimated purchased gilts demonstrated that 69.1% was detected positive in the second and/or the third samplings and none of them, including 130 doa purchased gilts, was positive prior to farrowing. In addition, 30.9% of the gilts was never detected positive by real time PCR during the study, suggesting a possible failure in the acclimation process, which could led to a future infection and shedding during the next farrowing. It is recognized that the identification of shedding seeders is an important step in gilt acclimation and it is recommended in order to guarantee a successful *M. hyopneumoniae* exposure.

Competing interests

The authors declare that they have no competing interest.

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References

-Amass, S.; Baysinger, A., 2006. Swine disease transmission and prevention. In: Straw, B.E.; Zimmerman, J.J.; Dállaire, S.; Taylor, D.J. (Eds.), Diseases of Swine. Nineth ed. Blackwell Publishing, Oxford, pp.1075-1098.

-Calsamiglia, M., Pijoan, C., Bosch, G. 1999. Profiling *Mycoplasma hyopneumoniae* in farms using serology and a nested PCR technique. Swine Health Prod. 7, 263-268.

-Calsamiglia, M., Pijoan, C. 2000. Colonization state and colostral immunity to *Mycoplasma hyopneumoniae* of different parity sows. Vet. Rec. 146, 530-532.

-Clark, L.K., Armstrong, C.H., Freeman, J.M., Scheidt, A.B., Knox, K., 1991. Investigating the transmission of *Mycoplasma hyopneumoniae* in a swine herd with enzootic pneumonia. Vet. Med. 86, 543-550.

- Dos Santos, L.F., Sreevatsan, S., Torremorell, M., Moreira, M.A.S., Sibila, M., Pieters, M., 2015. Genotype distribution of *Mycoplasma hyopneumoniae* in swine herds from different geographical regions. Vet. Microbiol.175, 374-381.

- Fano, E., Pijoan, C., Dee, S., Torremorell, M., 2006. Assessment of the effect of sow parity on the prevalence of *Mycoplasma hyopneumoniae* in piglets at weaning. Proceedings of the IPVS Congress, Copenhagen, Denmark, vol.1, p.96.

- Fano, E., Pijoan, C., Dee, S., Deen, J., 2007. Effect of *Mycoplasma hyopneumoniae* colonization at weaning on disease severity in growing pigs. Can. J. Vet. Res. 71, 195-200.

-Fablet, C., Marois, C., Kobish, M., Madec, F., Rose, N. 2010. Estimation of the sensitivity of four sampling methods for *Mycoplasma hyopneumoniae* detection in live pigs using a Bayesian approach. Vet. Microbiol. 143, 238-245.

-Kurth, K.T., Hsu, T., Snook, E.R., Thacker, E.L., Thacker, B.J., Minion, F.C. 2002. Use of a *Mycoplasma hyopneumoniae* nested polymerase chain reaction test to determine the optimal sampling sites in swine. J. Vet. Diagn. Invest.14, 463-469.

-Maes, D., Segales, J., Meyns, T., Sibila, M., Pieters, M., Haesebrouck, F. 2008. Control of *Mycoplasma hyopneumoniae* infection in pigs. Vet. Microbiol. 126, 297-309.

-Meyns, T., Maes, D., Dewulf, J., Vicca, J., Haesebrouck, F., De Kruif, A. 2004. Quantification of the spread of *Mycoplasma hyopneumoniae* in 52 nursery pigs using transmission experiments. Prev. Vet. Med. 66, 264-275.

-Morris, C.R., Gardner, I.A., Hietala, S.K., Carpenter, T.E., Anderson, R.J., Parker, K.M. 1995. Seroepidemiology study of natural transmission of *Mycoplasma hyopneumoniae* in a swine herd. Prev. Vet. Med. 21, 323-337.

- Pantoja, L.G., Pettit, K., Dos Santos, L.F., Tubbs, R., Pieters, M., 2016. *Mycoplasma hyopneumoniae* variability within a swine operation. J. Vet. Diagn. Invest. 28, 175–179.

- Pieters, M., Pijoa n, C., Fano, E., Dee, S. 2009. An assessment of the duration of *Mycoplasma hyopneumoniae* infection in an experimentally infected population of pigs. Vet. Microbiol. 134, 261-266.

- Pieters, M., Cline, G.S., Payne, B.J., Prado, C., Ertl, J.R., Rendahl, A.K., 2014. Intra-farm risk factors for *Mycoplasma hyopneumoniae* colonization at weaning age. Vet. Microbiol. 172, 575-580.

- Pieters, M.; Fano, E., 2016. *Mycoplasma hyopneumoniae* management in gilts. Vet. Rec. 178, 122-123.

-Pieters, M., Daniels, J., Rovira, A., 2017. Comparison of sample types and diagnostic methods for *in vivo* detection of *Mycoplasma hyopneumoniae* during early stages of infection. Vet. Microbiol. 203, 103-109.

-Pijoan, C., 2003. Prevalence of *Mycoplasma hyopneumoniae* in different parity sows as a preliminary tool for eradication. Research Report: Swine Health. 2, 1-5.

-Ramirez A., Karriker L. A., 2012. Herd Evaluation. In: Zimmerman J.J., Karriker, L.A., Ramirez A., Schwartz K.J., Stevenson G.W. (Eds.), Diseases of Swine. Tenth ed. Wiley-Blackwell, Ames, pp.15-17.

- R Core Team, 2015. R: A language and environment for statistical computing [Computer software]. R Foundation for Statistical Computing, Vienna, Austria. Available from http://www.r-project.org/.

-Roos, L.R., Fano, E., Homwong, N., Payne, B., Pieters, M. 2016. A model to investigate the optimal seeder-to-naïve ratio for successful natural *Mycoplasma hyopneumoniae* gilt exposure prior to entering the breeding herd. Vet. Microbiol. 184, 51-58.

-Sorensen, V., Ahrens, P., Barford, K., Feenstra, A.A., Feld, N.C., Friis, N.F., Bille-Hansen, V.B., Jensen, N.E., Pedersen, M.W. 1997. *Mycoplasma hyopneumoniae* infection in pigs: duration of the disease and evaluation of four diagnostic assays. Vet. Microbiol. 54, 23-34.

-Takeuti, K.L., Barcellos, D.E.S.N., Lara, A.C., Kunrath, C.F. 2017a. Detection of *Mycoplasma hyopneumoniae* in naturally infected gilts over time. Vet. Microbiol. 203, 215-220.

-Takeuti, K.L., Barcellos, D.E.S.N., Andrade, C.P., Almeida, L.L., Pieters, D. 2017b. Infection dynamics and genetic variability of *Mycoplasma hyopneumoniae* in self-replacement gilts. Vet. Microbiol. 208, 18-24.

-Thacker, E., 2006. Mycoplasmal diseases. In: Straw, B., Zimmerman, J., DAllaire, S., Taylor, D. (Eds.), Diseases of Swine. Ninth ed. Blackwell Publishing, Ames, pp. 701-717.

- Thacker, E.L., Minion, F.C., 2012. Mycoplasmosis. In: Zimmermann, J.J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G.W. (Eds.), Diseases of Swine. Tenth ed. Wiley-Blackwell, Ames, pp.779-797.

-Vicca, J., Stakenborg, T., Maes, D., Butaye, P., Peeters, J., Kruif, A., Haesebrouck, F. 2003. Evaluation of virulence of *Mycoplasma hyopneumoniae* field isolates. Vet. Microbiol. 97, 177-190.

- Vranckx, K., Maes, D., Calus, D., Villarreal, I., Pasmans, F., Hausebrouck, F., 2011. Multiple-locus variable-number tandem-repeat analysis is a suitable tool for differentiation of *Mycoplasma hyopneumoniae* strains without cultivation. J. Clin. Microbiol. 49, 2020–2023.

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Short communication

Mycoplasma hyopneumoniae detection in nylon-flocked and rayon-bud swabs☆,☆☆,☆☆☆

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ABSTRACT

The objective of this study was to compare the absorption and *M. hyopneumoniae* detection by real-time PCR using nylon-flocked and rayon-bud swabs. Results of this study showed that absorption and detection of *M. hyopneumoniae* were significantly higher in nylon-flocked swabs with Ct differences only ranging from 0.5 to 1.7.

Mycoplasma hyopneumoniae causes enzootic pneumonia (Goodwin et al., 1965; Mare and Switzer, 1965), a chronic respiratory condition affecting grow-finish pigs worldwide (Thacker and Minion, 2012). *M. hyopneumoniae* diagnosis can be accomplished using methods based on classic bacteriology, serology, histopathology, and molecular biology (Sibila et al., 2009). However, significant limitations in sensitivity and specificity of various diagnostic methods are widely recognized. For example, the bacterium is difficult to grow in laboratory conditions, antibodies production is delayed after infection and cannot be differentiated in vaccinated pigs, and lung lesions can only be assessed post-mortem, whilst not pathognomonic. On the other hand, PCR based testing exhibits high overall accuracy (Sibila et al., 2009) for *M. hyopneumoniae*, and infection can be detected even before the onset of clinical signs (Pieters and Pijoan, 2006) or seroconversion (Pieters et al., 2017). Various sample types can be collected from the respiratory tract of the pig to be tested by PCR. Among them, the use of sterile swabs to collect clinical samples is common (Kurth et al., 2002; Sibila et al., 2009; Flabet et al., 2010; Pieters et al., 2017).

Research studies have shown that respiratory pathogens can be detected using different types of swabs, and have suggested equal or higher sensitivity using flocked swabs over bud swabs (Daley et al., 2006; Esposito et al., 2010; Hernes et al., 2011; Ferguson-Noel et al., 2012). However, to the best of the author's knowledge, the effect of swab type on *M. hyopneumoniae* detection has not been evaluated. Therefore, the objective of this study was to compare two types of commercial swabs for *M. hyopneumoniae* detection by real-time PCR.

The first aim was to compare the absorption of nylon-flocked (FLOQSwabs™, Sparks, MD, USA) and rayon-bud swabs (BBL™ CultureSwab™, Sparks, MD). Ten nylon-flocked swabs and ten rayon-bud swabs were individually weighted and dipped into one matrix, either 200 µL of PBS or 0.600 g ± 0.002 g of lubricant, used as a surrogate for respiratory secretions (McKesson Medical-Surgical Inc., San Francisco, CA, USA). Swabs were dipped in the matrix for 30 s, rotating 5 times manually. Forty comparisons were made, corresponding to 10 repeats for each combination (type of swab and matrix). The second aim was to compare the detection of *M. hyopneumoniae* in the swabs by real-time PCR. Briefly, tracheal and bronchial mucous were collected from *M. hyopneumoniae* negative pigs after euthanasia and were tested to confirm the absence of the bacterium. A pure culture of *M. hyopneumoniae* strain AP414 (University of Minnesota) was mixed with the mucous secretions for a final concentration equivalent to1×10^5 CCU/mL, and was equally distributed into 40 aliquots (400 µL/tube). In each tube, one nylon-flocked and one rayon-bud swab were dipped for 10 s alternating the dipping order, as to avoid a confounding effect on the absorption of the material, increasing the performance of swabs dipped in first. Twenty repeats per swab type and dipping order were performed ($n = 80$). DNA extraction was performed using MagMAX™-96 Viral RNA isolation kit and MagMAX™ Express-96 Magnetic Particle Processor (Life Technologies, Grand Island, NY, USA). Samples were run by PCR using VetMAX™ qPCR Master mix and

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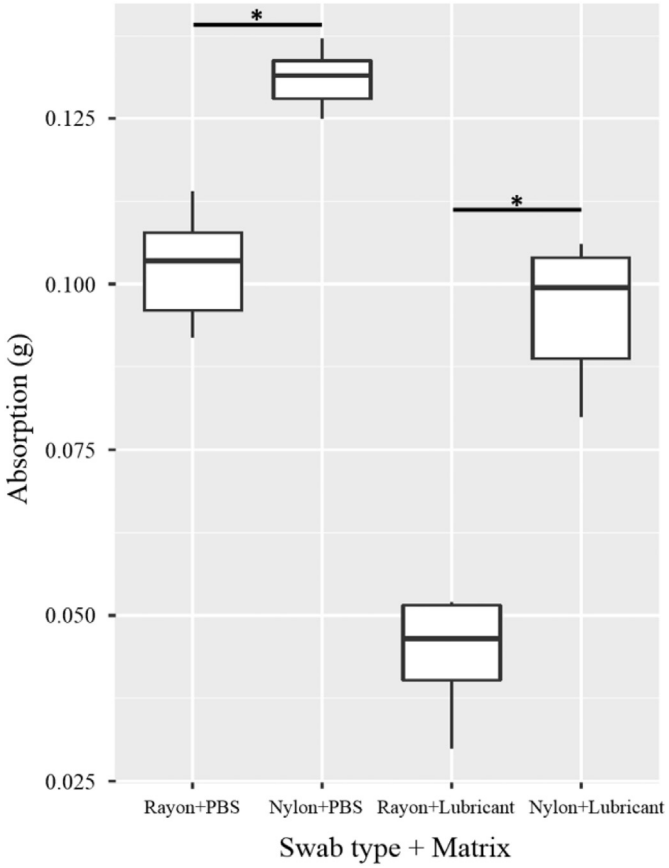

Fig. 1. Absorption of PBS or lubricant using different types of swabs (nylon-flocked and rayon-bud). Ten (10) measurements per swab type and matrix correspond to each box. The top and bottom lines of the box represent the first quartile and third quartile, respectively. The line inside the box represents the mean. The superior and inferior limits of the vertical lines represent the maximum and minimum values, respectively. Asterisks represent statistical differences ($p < 0.05$) between the two types of swabs using the same matrix.

VetMAX™ *M. hyopneumoniae* reagents kit (Life Technologies, Grand Island, NY, USA). Samples were tested individually in duplicate, with two positive and two negative controls. Positive controls consisted of extracted DNA of *M. hyopneumoniae* strain AP-414, whilst negative controls consisted of sterile molecular biology grade water. Paired two-tailed *t*-test was used to compare absorption of PBS or lubricant based on swab type, and a linear mixed model was performed to assess differences in Ct values between swab types (R v3.2; R Core Team, 2015). Significant differences were considered when *p-value* were < 0.05.

The absorption of PBS and lubricant was significant higher ($p < 0.05$) in nylon-flocked than in rayon-bud swabs (Fig. 1). Moreover, the comparison of the swab type using the same matrix revealed a higher difference between swabs when lubricant was used. Detection of *M. hyopneumoniae* by real-time PCR of each swab type is shown in Fig. 2. Overall, the mean Ct value of *M. hyopneumoniae* detection in nylon-flocked and rayon-bud swabs was significant different, and was significantly influenced by dipping order (mean 25.9; SD 0.48 and mean 26.4; SD 0.78 for nylon-flocked swabs, and mean 27.6; SD 0.84 and mean 26.9; SD 0.57 for rayon-bud swabs dipped first and second, respectively). No differences in the percent of positive samples detected by each swab type were observed, which could be attributed to the high bacterial load used for testing. Nevertheless, detection of positive samples using different swabs could be different under field conditions where bacterial loads may vary based on the phase of infection.

In other studies, significantly higher detection of respiratory pathogens or epithelial cells has been detected using nylon-flocked swabs over rayon-bud swabs (Daley et al., 2006; Esposito et al., 2010; Hernes et al., 2011). Specifically for Mycoplasma species, detection of *M. gallisepticum* and *M. synoviae* with different swab types has shown no significant differences in the percent of positive samples detected and mean Ct values (Ferguson-Noel et al., 2012). A mean decrease of 2.25 in the Ct value has been observed in nylon-flocked over rayon-bud swabs for viral detection in human patients with respiratory symptoms (Hernes et al., 2011). Similar Ct values were detected between flocked and bud swabs for Influenza detection in kids (Esposito et al., 2010).

In our study, PBS and lubricant absorption, and *M. hyopneumoniae* load were significantly higher in nylon-flocked swabs. In addition, the

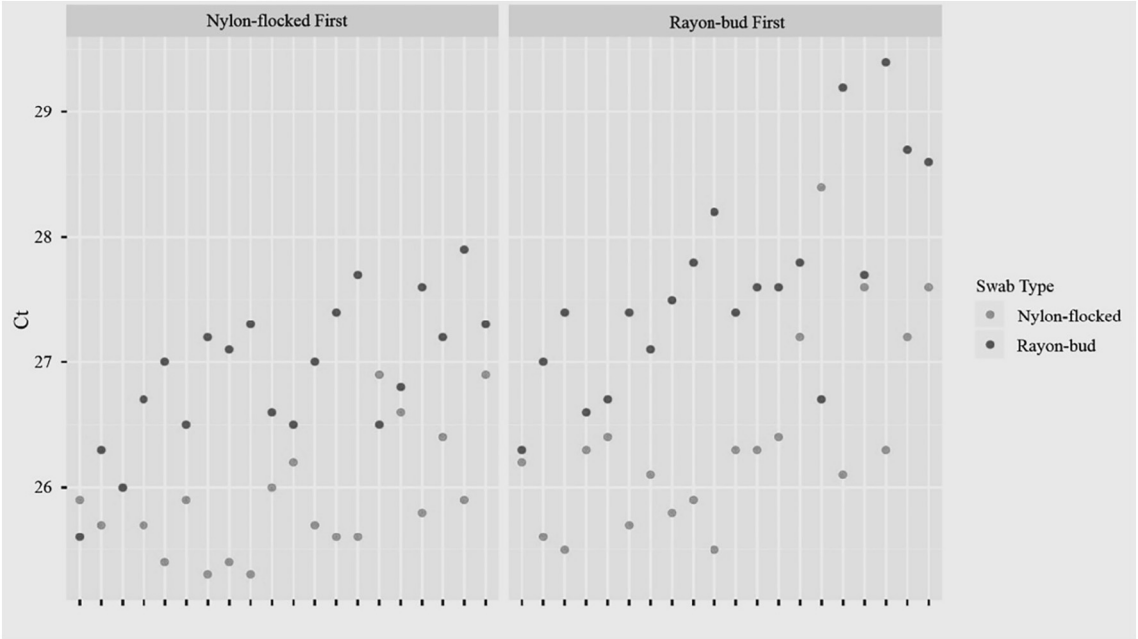

Fig. 2. *Mycoplasma hyopneumoniae* detection by real-time PCR. Samples were tested based on dipping order into a bacterial culture (panels) and on swab type (nylon-flocked and rayon-bud). All results are shown as Ct values. Gray dots: Nylon-flocked swabs. Black dots: Rayon-bud swabs. Each dot in each vertical line represents the mean Ct value of two real-time PCR runs of one aliquot of *M. hyopneumoniae* mixed with mucous secretions in which one nylon-flocked and one rayon-bud swab were dipped for 10 s. The two panels represent 40 aliquots run in duplicates.

mean Ct value of nylon-flocked swabs was lower than that of rayon-bud swabs, regardless of the dipping order. It is important to note that when rayon-bud swabs were dipped in the sample before nylon-flocked swabs, the average Ct value in nylon-flocked swabs was 0.5 higher, which might indicate that the rayon-bud swabs influenced the subsequent detection of *M. hyopneumoniae*, although the reason for this observation is uncertain.

Results of this study showed that absorption and detection of *M. hyopneumoniae* in nylon-flocked swabs was significantly higher, although Ct value differences were only 0.5 to 1.7, which may not be of significant importance from the biological and diagnostic perspective, as this difference translates into less than one log. Moreover, it is possible that variation in the absorption and detection of *M. hyopneumoniae* could occur in natural settings and maybe different from what was observed in this investigation, as the conditions of this *in vitro* experiment were artificially created. Nevertheless, it can be suggested that nylon-flocked swabs should be used in cases of *M. hyopneumoniae* chronic infections, when bacterial loads may be low and therefore more difficult to detect. Overall, our results highlight the influence of the material used for sampling for PCR detection.

References

Daley, P., Castriciano, S., Chernesky, M., Smieja, M., 2006. Comparison of flocked and rayon swabs for collection of respiratory epithelial cells from uninfected volunteers and symptomatic patients. J. Clin. Microbiol. 44, 2265–2267.

Esposito, S., Molteni, C.G., Daleno, C., Valzano, A., Cesati, L., Gualtieri, L., Tagliabue, C., Bosis, S., Principi, N., 2010. Comparison of nasopharyngeal nylon flocked swabs with universal transport medium and rayon-bud swabs with a sponge reservoir of viral transport medium in the diagnosis of paediatric influenza. J. Med. Microbiol. 59, 96–99.

Ferguson-Noel, N., Laibinis, V.A., Farrar, M., 2012. Influence of swab material on the detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* by real-time PCR. Avian Dis. 56, 310–314.

Flabet, C., Marois, C., Kobisch, M., Madec, F., Rose, N., 2010. Estimation of the sensitivity of four sampling methods for *Mycoplasma hyopneumoniae* detection in live pigs using a Bayesian approach. Vet. Microbiol. 143, 238–245.

Goodwin, R.F.W., Pomeroy, A.P., Whittlestone, P., 1965. Production of enzootic pneumonia in pigs with a mycoplasma. Vet. Rec. 77, 1247–1249.

Hernes, S.S., Quarsten, H., Hagen, E., Lyngroth, A.L., Pripp, A.H., Bjorvatn, B., Bakke, P.S., 2011. Swabbing for respiratory viral infections in older patients: a comparison of rayon and nylon flocked swabs. Eur. J. Microbiol. Infect. Dis. 30, 159–165.

Kurth, K.T., Hsu, T., Snook, E.R., Thacker, E.L., Thacker, B.J., Minion, F.C., 2002. Use of a *Mycoplasma hyopneumoniae* nested polymerase chain reaction test to determine the optimal sampling sites in swine. J. Vet. Diagn. Investig. 14, 463–469.

Mare, C.J., Switzer, W.P., 1965. New species: *Mycoplasma hyopneumoniae*; a causative agent of virus pig pneumonia. Vet. Med. Small Anim. Clin. 60, 841–846.

Pieters, M., Pijoan, C., 2006. Detection pattern of *Mycoplasma hyopneumoniae* DNA in nasal swabs. In: Proceedings of the International Pig Veterinary Society Congress, Copenhagen, Denmark, pp. 209.

Pieters, M., Daniels, J., Rovira, A., 2017. Comparison of sample types and diagnostic methods for in vivo detection of *Mycoplasma hyopneumoniae* during early stages of infection. Vet. Microbiol. 203, 103–109.

R Core Team, 2015. R: A Language and Environment for Statistical Computing [Computer Software]. R Foundation for Statistical Computing. http://www.r-project.org/.

Sibila, M., Pieters, M., Molitor, T., Maes, D., Haesebrouck, F., Segalés, J., 2009. Current perspectives on the diagnosis and epidemiology of *Mycoplasma hyopneumoniae* infection. Vet. J. 181, 221–231.

Thacker, E.L., Minion, F.C., 2012. Mycoplasmosis. In: Zimmermann, J.J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G.W. (Eds.), Diseases of Swine, Tenth ed. Wiley-Blackwell, Ames, pp. 779–797.

7. CONSIDERAÇÕES FINAIS

A dinâmica de infecção de *M. hyopneumoniae* nos planteis de suínos tem sido amplamente estudada pela importância deste patógeno na suinocultura e pelas dificuldades no controle da infecção. As leitoas e matrizes de menor ordem de parto possuem papel importante na transmissão de *M. hyopneumoniae*, visto que podem eliminar a bactéria durante o parto ou no período lactacional, colonizando seus leitões. O contato entre leitões infectados e suscetíveis pode acarretar na disseminação da infecção no plantel e na manifestação de sinais clínicos respiratórios durante a fase de creche ou terminação. Sabendo-se da importância das leitoas na dinâmica de infecção do *M. hyopneumoniae*, este projeto teve como objetivo avaliar a detecção longitudinal deste agente em leitoas de reposição em três experimentos.

Os resultados do primeiro experimento indicaram que *M. hyopneumoniae* é normalmente detectado por PCR por um a três meses em infecções naturais, embora eventuais detecções por até cinco meses possam ser encontradas. Ainda, a presença de subpopulações negativas para *M. hyopneumoniae* foi demonstrada pela primeira vez em granjas positivas para o agente.

No segundo experimento, a detecção de *M. hyopneumoniae* em leitoas de reposição interna demonstrou que a maioria das leitoas são detectadas positivas para *M. hyopneumoniae* por PCR quando alojadas nas granjas (150 dias de idade) e que elas podem eliminar o agente próximo ao parto. Além disso, a ausência de detecção de *M. hyopneumoniae* em uma quantidade considerável de leitoas durante o experimento pode indicar que ainda sejam suscetíveis à infecção após o primeiro parto, evidenciando a importância da aclimatação mesmo em leitoas de reposição interna. Ainda, ampla variabilidade genética de *M. hyopneumoniae* foi detectada entre granjas após avaliação por MLVA.

No terceiro estudo, a detecção de *M. hyopneumoniae* em leitoas de reposição externa (negativas para *M. hyopneumoniae*) introduzidas em granjas positivas foi semelhante independentemente do tipo de fluxo de aclimatação de leitoas (contínuo ou *all-in all-out*). Ainda, uma quantidade expressiva de leitoas

nunca foi detectada positiva para *M. hyopneumoniae*, sugerindo que a aclimatação pode apresentar falhas, o que acarretaria em uma possível susceptibilidade dessas leitoas a uma futura exposição à bactéria. A variabilidade genética de *M. hyopneumoniae* também foi avaliada por MLVA e verificou-se um reduzido número de variantes nos três planteis.

Um quarto experimento foi realizado para avaliar dois tipos de suabes na detecção de *M. hyopneumoniae*. Os resultados deste estudo indicaram que suabes de nylon flocados apresentam uma maior absorção de material e maior sensibilidade na detecção de *M. hyopneumoniae* quando comparados a suabes de ponta de rayon, podendo ser utilizados em situações nas quais uma baixa quantidade de bactérias está presente, como em infecções crônicas.

Os resultados deste estudo contribuem para uma melhor compreensão da dinâmica de infecção de *M. hyopneumoniae* em leitoas de reposição, as quais desempenham papel importante na transmissão da bactéria. Além disso, as informações geradas nestes estudos podem ser utilizadas a campo para auxiliar no estabelecimento de medidas de controle mais eficazes.

8. REFERÊNCIAS BIBLIOGRÁFICAS

ABPA (Associação Brasileira de Proteína Animal), 2015.

AMASS, S. F.; CLARK, L. K.; VAN ALSTINE, W. G.; BOWERSOCK, T. L.; MURPHY, D. A.; KNOX, K. E.; ALBREGTS, S. R. Interaction of *Mycoplasma hyopneumoniae* and *Pasteurella multocida* infections in swine. **Journal of the American Veterinary Medical Association**, v.204, n.1, p.102-7, 1994.

AMASS, S.; BAYSINGER, A. **Swine disease transmission and prevention**. In: Diseases of Swine. STRAW, B. E.; ZIMMERMAN, J. J.; DÁLLAIRE, S.; TAYLOR, D. J. 9. ed. Oxford: Blackwell Publishing, p.1075-1098, 2006.

ANDERSON, A.; DALQUIST, L.; LEUWERKE, B.; SPONHEIM, A.; FANO, E.; PIETERS, M. Improvement of *Mycoplasma hyopneumoniae* culture and isolation focusing on sample selection and handling. In: **Proceedings of the Leman Swine Conference**, Saint Paul, 2016.

ARSENAKIS, I.; MICHIELS, A.; DEL POZO SACRISTÁN, R.; BOYEN, F.; HAESEBROUCK, F.; MAES, D*. Mycoplasma hyopneumoniae* vaccination at or shortly before weaning under field conditions: a randomised efficacy trial. **Veterinary Record**, v.181, p.1-9, 2017.

BACCARO, M. R.; HIROSE, F.; UMEHARA, O.; GONÇALVES, L. C.; DOTO, D. S.; PAIXÃO, R.; SHINYA, L. T.; MORENO, A. M. Comparative efficacy of two single-dose bacterins in the control of *Mycoplasma hyopneumoniae* in swine raised under commercial conditions in Brazil. **Veterinary Journal**, v.172, n.3, p.526-31, 2006.

BANDRICK, M.; PIETERS, M.; PIJOAN, C.; BAIDOO, S. K.; MOLITOR, T. W. Effect of cross-fostering on transfer of maternal immunity to *Mycoplasma hyopneumoniae* to piglets. **Veterinary Record,** v. 168, n.4, p.100-105, 2011.

BARCELLOS, D. E. S. N.; BOROWSKI, S. M.; GHELLER, N. B.; SANTI, M.; MORES, T. J. Relação entre manejo, ambiente e doenças respiratórias em suínos. **Acta Scientiae Veterinariae**, v.36, n.1, p.87-93, 2008.

BARCELLOS, D. E. S. N. **Informação pessoal**, 2017.

BLANCHARD, B.; VENA, M. M.; CAVALIER, A.; LE LANNIC, J.; GOURANTON, J.; KOBISH, M. Electron microscopic observations of the respiratory tract of SPF piglets inoculated with *Mycoplasma hyopneumoniae*. **Veterinary Microbiology**, v.30, n.4, p.329-41, 1992.

BROWNE, C.; LOEFFLER, A.; HOLT, H.; CHANG, Y. M.; LLOYD, D. H.; NEVEL, A. Low temperature and dust favour in vitro survival of *Mycoplasma hyopneumoniae*: time to revisit indirect transmission in pig housing. **Letters in Applied Microbiology**, v. 64, n.1, 2-7, 2017.

BROWNING, G. F.; MARENDA, M. S.; MARKHAM, P. F.; NOORMOHAMMADI, A. H.; WHITHEAR, K. G. **Mycoplasma**. In: Pathogenesis of Bacterial Infections in Animals. GYLES, C. L.; PRESCOTT, J. F.; SONGER, J. G.; THOEN, C. O. 4. ed. Oxford: Blackwell Publishing, p.549-566, 2010.

BURCH, D. G. S. The comparative efficacy of antimicrobials for the prevention and treatment of enzootic pneumonia and some of their pharmacokinetic/pharmacodynamics relationships. **The Pig Journal**, v.53, p.8-27, 2004.

CALSAMIGLIA, M.; PIJOAN, C.; BOSCH, G. Profiling *Mycoplasma hyopneumoniae* in farms using serology and a nested PCR technique. **Swine Health and Production**, v.7, n.6, p.263-268, 1999a.

CALSAMIGLIA, M.; PIJOAN, C.; TRIGO, A. Application of a nested polymerase chain reaction assay to detect *Mycoplasma hyopneumoniae* from nasal swabs. **Journal of Veterinary Investigation**, v.11, p.246-251, 1999b.

CALSAMIGLIA, M.; PIJOAN, C. Colonisation state and colostral immunity to *Mycoplasma hyopneumoniae* of different parity sows. **Veterinary Record**, v. 146, p.530-532, 2000.

CLARK, L. K.; ARMSTRONG, C. H.; FREEMAN, J. M. SCHEIDT, A. B.; KNOX, K. Investigating the transmission of *Mycoplasma hyopneumoniae* in a swine herd with enzootic pneumonia. **Veterinary Medicine**, v.86, p.543-550, 1991.

DEBLANC, C.; GORIN, S.; QUÉGUINER, S.; GAUTIER-BOUCHARDON, A. V.; FERRÉ, S.; AMENNA, N.; CARIOLET, R.; SIMON, G. Pre-infection of pigs with *Mycoplasma hyopneumoniae* modifies outcomes of infection with European swine influenza virus of H1N1, but not H1N2, subtype. **Veterinary Microbiology**, v.157, p.96-105, 2012.

DEBLANC, C.; DELGADO-ORTEGA, M.; GORIN, S.; BERRI, M.; PABOEUF, F.; BERTHON, P.; HERRLER, G,; MEURENSM F.; SIMON, G. *Mycoplasma hyopneumoniae* does not affect the interferon-related anti-viral response but predisposes the pig a higher level of inflammation following swine influenza vírus infection. **Journal of General Virology**, v.97, p.2501-2515, 2016.

DEEGAN, C.; DALQUIST, L.; PIETERS, M. Identification of *Mycoplasma hyopneumoniae* shedding gilts in the field. In: **Proceedings of the XLVII Annual Meeting of The American Association of Swine Veterinarians**, New Orleans, p.234-235, 2016.

DESROSIERS, R. A review of some aspects of the epidemiology, diagnosis, and control of *Mycoplasma hyopneumoniae* infections. **Journal of Swine Health and Production**, v.9, n.5, p.233-237, 2001.

DOS SANTOS, J. L.; DOS SANTOS, L. F.; MATOS, M. C.; SOBESTIANSKY, J.; BARCELLOS, D. **Micoplasmoses**. In: Doenças dos Suínos.

SOBESTIANSKY, J.; BARCELLOS, D. 2. ed. Goiânia: Cânone Editorial, p.216-229, 2012.

DOS SANTOS, L. F.; SREEVATSAN, S.; TORREMORELL, M.; MOREIRA, M. A. S.; SIBILA, M.; PIETERS, M. Genotype distribution of *Mycoplasma hyopneumoniae* in swine herds from different geographical regions. **Veterinary Microbiology**, v.175, p.374-381, 2015.

DRITZ, S. S.; CHENGAPPA, M. M.; NELSSEN, J. L.; TOKACH, M. D.; GOODBAND, R.D.; NIETFELD, J. C.; STAATS, J.J. Growth and microbial flora of nonmedicated, segregated, early weaned pigs from a commercial swine operation. **Journal of American Veterinary Medical Association**, v.208, n.5, p.711-5, 1996.

FANO, E.; PIJOAN, C.; DEE, S. Dynamics and persistence of *Mycoplasma hyopneumoniae* infection in pigs. **The Canadian Journal of Veterinary Research**, v.69, p. 223-228, 2005.

FANO, E. A.; PIJOAN, C.; DEE, S. A. Assessing the *Mycoplasma hyopneumoniae* infection pattern in a sow herd following an increase in the replacement rate. In: **Proceedings of the XIX IPVS Congress**, Copenhagen, p.135, 2006a.

FANO, E.; PIJOAN, C.; DEE, S.A.; TORREMORELL, M. Assessment of the effect of sow parity on the prevalence of *Mycoplasma hyopneumoniae* in piglets at weaning. In: **Proceedings of the XIX IPVS Congress**, Copenhagen, p.96, 2006b.

FANO, E.; PIJOAN, C.; DEE, S.; DEEN, J. Effect of *Mycoplasma hyopneumoniae* colonization at weaning on disease severity in growing pigs. **The Canadian Journal of Veterinary Research**, v.71, p.195-200, 2007.

FLABET, C.; MAROIS, C.; KOBISCH, M.; MADEC, F.; ROSE, N. Estimation of the sensitivity of four sampling methods for *Mycoplasma hyopneumoniae* detection in live pigs using a Bayesian approach. **Veterinary Microbiology**, v.143, p.238-245, 2010.

FRIIS, N. F. Resistance of porcine mycoplasmas to heat. **Acta Veterinaria Scandinavica**, v. 15, p. 283-285, 1974.

FRIIS, N. F. Some recommendation concerning primary isolation of *Mycoplasma suipneumoniae* and *Mycoplasma flocculare*. **Nord Veterinary Medicine**, v.27, p.337-339, 1975.

GIACOMINI, E.; FERRARI, N.; PITOZZI, A.; REMSTANI, M.; GIARDIELLO, D.; MAES, D.; ALBORALI, G. L. Dynamics of *Mycoplasma hyopneumoniae* seroconversion and infections in pigs in the three main production systems. **Veterinary Research Communications**, v.40, p.81-88, 2016.

GOODWIN, R. F. W. The phenomenon of suppressed respiratory disease in the litters of older sows. **Veterinary Research**, v.77, n.14, p.383-387, 1965.

HAESEBROUCK, F.; PASMANS, F.; CHIERS, K.; MAES, D.; DUCATELLE, R.; DECOSTERE, A. Efficacy of vaccines against bacterial diseases in swine: what can we expect? **Veterinary Microbiology**, v.100, p.255-268, 2004.

HILL, M. A.; SCHEIDT, A. B.; TECLAW, R. F.; CLARK, L. K.; KNOX, K. E.; JORDAN, M. Association between growth indicators and volume of lesions in lugs from pigs at slaughter. **American Journal of Veterinary Research**, v.53, p.2221-2223, 1992.

HOLST, S.; YESKE, P.; LEUWERKE, B.; SWALLA, R.; DAVIES, P.; PIETERS, M. Effect of pre-farrow administration of tulathromycin injectable solution on *Mycoplasma hyopneumoniae* prevalence in suckling pigs at birth and weaning. In: **Proceedings of the XLIII Annual Meeting of The American Association of Swine Veterinarians**, San Diego, p.89-90, 2013.

HOLST, S.; YESKE, P.; PIETERS, M. Elimination of *Mycoplasma hyopneumoniae* from breed-to-wean farms: a review of current protocols with emphasis on herd closure and medication. **Journal of Swine Health and Production**, v.23, n.6, p.321-330, 2015.

HUTCHISON, C. III.; MONTAGNE, M. Mycoplasmas and the Minimal Genome Concept. **Molecular Biology and Pathogenicity of Mycoplasmas**, p.221-253, 2002.

IBGE (Instituto Brasileiro de Geografia e Estatística), 2015.

KICH, J. D.; KUCHIISHI, S. S.; MORES, M. A. Z.; LARA, A. C. Agentes bacterianos de pneumonia associados a infecção por *Mycoplasma hyopneumoniae*. **Acta Scientiae Veterinariae**, v.38, n.1, p.17-27, 2010.

LE GRAND, A.; KOBISCH, M. Comparison de l'utilisation d'un vaccine te d'un traitement antibiotique séquentiel dans un élevage infecté par *Mycoplasma hyopneumoniae*. **Veterinary Medicine**, v.27, p.241-253, 1996.

LIN, J. H.; CHEN, S.P.; YEH, K. S.; WENG, C. N. *Mycoplasma hyorhinis* in Taiwan: diagnosis and isolation of swine pneumonia pathogen. **Veterinary Microbiology**, v.115, p.111-116, 2006.

LÓPEZ, A. **Respiratory System**. In: Pathologic Basis of Veterinary Disease. MCGAVIN, M. D.; ZACHARY, J. F. St. Louis: Mosby Elsevier, p. 535-541, 2007.

LOWE, J. F. *Mycoplasma hyopneumoniae*: gilts, are they the problem? In: **Proceedings of the Allen D. Leman Swine Conference**, St. Paul, p.83-85, 2012.

MAES, D.; DELUYKER, H.; VERDONCK, M.; CASTRYCK, F.; MIRY, C.; LEIN, A.; VRIJENS, B.; KRUIF, A. The effect of vaccination against *Mycoplasma*

hyopneumoniae in pig herds with a continuous production system. **Journal of Veterinary Medicine**, v.45, p.495-505, 1998.

MAES, D.; DELUYKER, H.; VERDONCK, M.; CASTRYCK, F.; MIRY, C.; VRIJENS, B.; VERBEKE, W.; VIAENE, J.; DE KRUIF, A. Effect of vaccination against *Mycoplasma hyopneumoniae* in pig herds with all-in/all-out production system. **Vaccine**, v.17, p.1024-1034, 1999.

MAES, D.; SEGALES, J.; MEYNS, T.; SIBILA, M.; PIETERS, M.; HAESEBROUCK, F. Control of *Mycoplasma hyopneumoniae* infections in pigs. **Veterinary Microbiology**, v.126, p.297-309, 2008.

MAES, D.; PASMANS, F.; HAESEBROUCK, F. Porcine Mycoplasmas: the never ending story. In: **Proceedings of the V Asian Pig Veterinary Society Congress**, Pattaya, p.7-15, 2011.

MARKEY, B.; LEONARD, F.; ARCHAMBAULT, M.; CULLINANE, A.; MAGUIRE, D. **Clinical Veterinary Microbiology**. 2. ed. Toronto: Mosby Elsevier, 901 p., 2013.

MAROIS, C.; DORY, D.; FABLET, C.; MADEC, F.; KOBISCH, M. Development of a quantitative Real-Time TaqMan PCR assay for determination of the minimal dose of *Mycoplasma hyopneumoniae* strain 116 required to induce pneumonia in SPF pigs. **Journal of Applied Microbiology**, v.108, p.1523-1533, 2010.

MATEUSEN, B.; MAES, D.; VAN GOUBERGEN, M.; VERDONCK, M.; DE KRUIF, A. Effectiveness of treatment with lincomycin hydrochloride and/or vaccination against *Mycoplasma hyopneumoniae* for controlling chronic respiratory disease in a herd of pigs. **Veterinary Record**, v.151, p.135-140, 2002.

MEYNS, T.; MAES, D.; DEWULF, J.; VICCA, J.; HAESEBROUCK, F.; DE KRUIF, A. Quantification of the spread of *Mycoplasma hyopneumoniae* in nursery pigs using transmission experiments. **Preventive Veterinary Medicine**, v.66, p.264-275, 2004.

MEYNS, T.; DEWULF, J.; DE KRUIF, A.; CALUS, D.; HAESEBROUCK, F.; MAES, D. Comparison of transmission of *Mycoplasma hyopneumoniae* in vaccinated and non-vaccinated populations. **Vaccine**, 24, p.7081-7086, 2006.

MICHIELS, A.; VRACKX, K.; PIEPERS, S.; SACRISTÁN, R. D. P.; ARSENAKIS, I.; BOYEN, F.; HAESEBROUCK, F.; MAES, D. Impact of diversity of *Mycoplasma hyopneumoniae* strains on lung lesions in slaughter pigs. **Veterinary Research**, v. 48, n.2, p.1-14, 2017.

MINION, F. C.; LEFKOWITZ, E. J.; MADSEN, M. L.; CLEARY, B. J.; SWARTZELL, S. M.; MAHAIRAS, G. G. The genome sequence of *Mycoplasma hyopneumoniae* strain 232, the agent of swine mycoplasmosis. **Journal of Bacteriology**, v.186, n.21, p.7123-7133, 2004.

MOREIRA, N. M.; MATOS, M. P. C.; MOURA, V. M. B. D.; AMARAL, H. R. D.; SOBESTIANSKY, J. **Monitoramento de lesões pulmonares e hepáticas de suínos abatidos em Goiânia-Goiás**. 2011. Disponível em: http://www.sbpcnet.org.br/livro/63ra/conpeex/pibic/trabalhos/NAT__LIA.PDF.

MORES, M. A. Z. **Pneumonias em suínos**. In: Tópicos em sanidade e manejo de suínos. ALFIERI, A. F.; BARRY, A. F.; ALFIERI, A. A.; SILVA, C. A.; DALLANORA, D.; ZOTTI, E.; ALBERTON, G. C.; RODRIGUES, I. M. T. C.; MACHADO, I. P.; GRIESSLER, K.; MORES, M. A. Z.; DITTRICH, R. L.; STARKL, V. Sorocaba: Curuca Consciência Ecológica, p.19-67, 2010.

MORRIS, C. R.; GARDNER, I. A.; HIETALA, S. K.; CARPENTER, T. E.; ANDERSON, R. J.; PARKER, K. M. Persistence of passively acquired antibodies to *Mycoplasma hyopneumoniae* in a swine herd. **Preventive Veterinary Medicine**, v.21, p.29-41, 1994.

NEUMANN, E. J.; RAMIREZ, A.; SCHWARTZ, K. J. **Mycoplasmal Pneumonia (Enzootic Pneumonia)**. In: Swine disease manual. 4. ed. Perry: American Association of Swine Veterinarians, p.29-30, 2009.

OTAKE, S.; DEE, S.; CORZO, C.; OLIVEIRA, S.; DEEN, J. Long-distance airborne transport of infectious PRRSV and *Mycoplasma hyopneumoniae* from a swine population infected with multiple viral variants. **Veterinary Microbiology**, v.145, p.198-208, 2010.

PAINTER, T.; KUHN, M.; WOLFF, T.; PIETERS, M.; SENN, M. 2012. Efficacy and duration of infection study for Respisure and Draxxin against a *Mycoplasma hyopneumoniae* challenge in swine**.** In: **Proceedings of the XXII IPVS Congress**, Jeju, p.207, 2012.

PANTOJA, L. G.; PETTIT, K.; DOS SANTOS, L. F.; TUBBS, R.; PIETERS, M. *Mycoplasma hyopneumoniae* genetic variability within a swine operation. **Journal of Veterinary Diagnostic Investigation**, v. 28, n.2, p.175-179, 2016.

PARK, S. C.; YIBCHOK-ANUN, S.; CHENG, H.; YOUNG, T. F.; TACKER, E. L.; MINION, F. C.; ROSS, R. F.; HSU, W. H. *Mycoplasma hyopneumoniae* increased intracellular calcium release in porcine ciliated tracheal cells. **Infection and Immunity**, v.70, p.2502-2506, 2002.

PARK, C.; JEONG, J.; CHOI, K.; CHAE, C. Efficacy of a new bivalent vaccine of porcine circovirus type 2 and *Mycoplasma hyopneumoniae* (Fostera PCV MH) under experimental conditions. **Vaccine**, v.34, p.270-275, 2016a.

PARK, C.; JEONG, J.; KANG, I.; CHOI, K.; PARK, S. J.; CHAE, C. Increased fucosyl glycoconjugate by *Mycoplasma hyopneumoniae* enhances adherences of *Pasteurella multocida* type A in the ciliated epithelial cells of the respiratory tract. **Veterinary Research**, v.12, n.25, p.1-6, 2016b.

PIETERS, M.; PIJOAN, C. Detection pattern of *Mycoplasma hyopneumoniae* DNA in nasal swabs. In: **Proceedings of the International Pig Veterinary Society Congress**, Copenhagen, p.209, 2006.

PIETERS, M.; PIJOAN, C.; FANO, E.; DEE, S. An assessment of the duration of *Mycoplasma hyopneumoniae* infection in an experimentally infected population in pigs. **Veterinary Microbiology**, v.134, p.261-266, 2009.

PIETERS, M.; CLINE, G. S.; PAYNE, B. J.; PRADO, C.; ERTL, J. R.; RENDAHL, A. K. Intra-farm risk factors for *Mycoplasma hyopneumoniae* colonization at weaning age. **Veterinary Microbiology**, v.172, p.575-580, 2014.

PIETERS, M.; FANO, E. *Mycoplasma hyopneumoniae* management in gilts. **Veterinary Record**, 178, p.122-123, 2016.

PIETERS, M.; DANIELS, J.; ROVIRA, A. Comparison of sample types and diagnostic methods for in vivo detection of *Mycoplasma hyopneumoniae* during early stages of infection. **Veterinary Microbiology**, v.203, p.103-109, 2017.

PIETERS, M.; SIBILA, M. When is the best time to vaccinate piglets against *Mycoplasma hyopneumoniae*. **Veterinary Record**, v.181, p.16-17, 2017.

PIJOAN, C. Prevalence of *Mycoplasma hyopneumoniae* in different parity sows as a preliminary tool for eradication. **Research Report: Swine Health**, v.2, n.81, p.1-5, 2003.

RAZIN, S.; YOGEV, D.; NAOT, Y. Molecular biology and pathogenicity of mycoplasmas. **Microbiology and Molecular Biology Reveviews**, v. 62, p.1094-1156, 1998.

REDONDO, E.; MASOT, A. J.; FERNÁNDEZ, A.; GÁZQUEZ, A. Histopathological and immunohistochemical findings in the lungs of pigs infected experimentally with *Mycoplasma hyopneumoniae*. **Journal of Comparative Pathology**, v.140, p.260-270, 2009.

REIS, R.; LEMOS, J.M.; CAVALCANTE, J.E. Estudo das lesões pulmonares de suínos de abate. **Arquivos Brasileiros de Medicina Veterinária e Zootecnia**. v.44, n.5, p.407-418, 1992.

ROCHA, E. P.; BLANCHARD, A. Genomic repeats, genome plasticity and the dynamics of Mycoplasma evolution. **Nucleic Acids Research**, v.30, p.2031-2042, 2002.

ROOS, L. R.; FANO, E.; HOMWONG, N.; PAYNE, B.; PIETERS, M. A model to investigate the optimal seeder-to-näive ratio for successful natural *Mycoplasma hyopneumoniae* gilt exposure prior to entering the breeding herd. **Veterinary Microbiology**, v.184, p.51-58, 2016.

RUIZ, A.; UTRERA, V.; PIJOAN, C. Effect of *Mycoplasma hyopneumoniae* sow vaccination on piglet colonization at weaning. **Journal of Swine Health and Production**, v.11, p.131-135, 2003.

SCHWARTZ, K. Current trends and diagnostic tools. **A contemporary review of *Mycoplasma hyopneumoniae* control strategies**, p.11-16, 2016.

SIBILA, M.; CALSAMIGLIA, M.; VIDAL, D.; BADIELLA, L.; ALDAZ, A.; JENSEN, J.C. Dynamics of *Mycoplasma hyopneumoniae* infection in 12 farms with different production systems. **The Canadian Journal of Veterinary Research**, v.68, p.12-18, 2004.

SIBILA, M.; BERNAL, R.; TORRENT, D.; LLOPART, D.; RIERA, P.; CALSAMIGLIA, M. Effect of *Mycoplasma hyopneumoniae* sow vaccination on colonization, seroconversion and presence of enzootic pneumonia compatible lung lesions. In: **Proceedings of the XIX IPVS Congress**, Copenhagen, p.103, 2006.

SIBILA, M.; NOFRARIAS, M.; LÓPEZ-SORIA, S.; SEGALÉS, J.; RIERA, P.; LLOPART, D.; CALSAMIGLIA, M. Exploratory field study on *Mycoplasma hyopneumoniae* infection in suckling pigs. **Veterinary Microbiology**, v.121, p.352-356, 2007.

SIBILA, M.; PIETERS, M.; MOLITOR, T.; MAES, M.; HAESEBROUCK, F.; SEGALES, J. Current perspectives on the diagnosis and epidemiology of *Mycoplasma hyopneumoniae infection*. **Veterinary Journal**, v.181, p.221-231, 2009.

SIEVERS, C.; DALQUIST, L.; SPONHEIM, A.; FANO, E.; WETZELL, T. Comparison of ante-mortem sampling procedures to post-mortem bronchial sampling for the detection of *Mycoplasma hyopneumoniae* by PCR. In: **Proceedings of the XLVI Annual Meeting of The American Association of Swine Veterinarians**, Orlando, p.115-116, 2015.

SILVA, A.; PAGANINI, F.; ACOSTA, J.; ROCHA, P.; MISTURA, H.; MARCON, E.; SIMON, V.; CASAGRANDE, H. Programa de gerenciamento de doenças respiratórias em suínos. I - Estudo do perfil das doenças respiratórias nas regiões Sul, Sudeste e Centro-oeste do Brasil. In: **Anais da X ABRAVES**, Porto Alegre, p.31-32, 2001.

SILVA, A. F.; PAGANINI, F. J.; ACOSTA, J. C.; ROCHA, P. H.; MISTURA, H.; MARCON, E.; SIMON, V.A.; CASAGRANDE, H.A.; SILVA, M.S.; FLOSS, J. M. Prevalence of respiratory diseases in swine at slaughterhouses in Brasil. In: **Proceedings of the XVII IPVS Congress**, Ames, p.332, 2002.

SILVA, A.F.; SILVA, M.; ACOSTA, J.C.B.; OLIVEIRA, H.; BURCIUS, L.; ROCHA, F. Avaliação do comprometimento pulmonar em suínos das regiões Sul, Sudeste e Centro-Oeste do Brasil pelo emprego do programa de gerenciamento da saúde respiratória em suínos (PEC) de 2002 a 2006. In:

Anais do Congresso Latino-Americano de Suinocultura, Foz do Iguaçu, p.429-432, 2006.

SOBESTIANSKY, J.; PIFFER, I. A.; DE FREITAS, A. R. Impacto de doenças respiratórias dos suínos nos sistemas de produção do estado de Santa Catarina. **Comunicado Técnico Embrapa Suínos e Aves**, p.1-4, 1987.

SOBESTIANSKY, J.; DALLA COSTA, O.; MORES, N.; BARIONI, W. JR.; PIFFER, I.; PEDROSO -DE-PAIVA, D.; Estudos ecopatológicos nas fases de crescimento e terminação: prevalência de rinite atrófica e de pneumonia nas fases de crescimento e terminação na região sul do Brasil. In: **Anais da IX ABRAVES**, Belo Horizonte, p.171- 172, 1999.

SOBESTIANSKY, J.; COSTA, O. A. D.; MORÉS, N.; BARIONI JUNIOR, W.; PIFFER, I. A.; GUZZO, R. Estudos ecopatológicos das doenças respiratórias dos suínos: prevalência e impacto econômico em sistemas de produção dos estados de Santa Catarina, Rio Grande do Sul e Paraná. **Comunicado Técnico Embrapa Suínos e Aves**, p.1-6, 2001.

SORENSEN, V.; AHRENS, P.; BARFORD, K.; FEENSTRA, A. A.; FELD, N. C.; FRISS, N. F.; BILLE-HANSEN, V. B.; JENSE, N. E.; PEDERSEN, M. W. *Mycoplasma hyopneumoniae* infection in pigs: duration of the disease and evaluation of four diagnostic assays. **Veterinary Microbiology**, v.54, p.23-34, 1997.

STEPAN, A. L. S. Tipificação e sensibilidade de amostras de *Pasteurella multocida* isoladas a partir de lesões de pleurite em suínos terminados. 1995. Porto Alegre, **Dissertação de Mestrado**, Curso de Pós-Graduação em Ciências Veterinárias, Universidade Federal do Rio Grande do Sul.

TAKEUTI, K. L.; WATANABE, T. T. N.; DE CASTRO, L. A.; DRIEMEIER, D.; BARCELLOS, D. E. S. N. Caracterização histopatológica e imuno-histoquímica da pneumonia causada pela co-infecção por *Pasteurella multocida* e *Mycoplasma hyopneumoniae* em suínos. **Acta Scientiae Veterinariae**, v.41, p.1-6, 2013.

THACKER, E.; THACKER, B.; BOETTCHER, T.; JAYAPPA, H. Comparison of antibody production, lymphocyte stimulation and production induced by four commercial *Mycoplasma hyopneumoniae* bacterins. **Swine Health and Production**, 6, p.107-12, 1998.

THACKER, E. L.; THACKER, B. J.; KUHN, M.; HAWKINS, P. A.; WATERS, W. R. Evaluation of local and systemic immune responses induced by intramuscular injection of a *Mycoplasma hyopneumoniae* bacterin to pigs. **American Journal of Veterinary Research**, v.61, n.11, p.1384-1389, 2000.

THACKER, E. L.; MINION, F. C. **Mycoplasmosis**. In: Diseases of Swine. ZIMMERMANN, J. J.; KARRIKER, L. A.; RAMIREZ, A.; SCHWARTZ, K. J.; STEVENSON, G. W. 10. ed. Ames: Wiley-Blackwell, p.779-797, 2012.

VICCA, J.; STAKENBORG, T.; MAES, D.; BUTAYE, P.; PEETERS, J.; KRUIF, A.; HAESEBROUCK, F. Evaluation of virulence of *Mycoplasma hyopneumoniae* field isolates. **Veterinary Microbiology**, v.97, p.177-190, 2003.

VICCA, J.; STAKENBORG, R.; MAES, D.; BUTAYE, P.; PEETERS, J.; DE KRUIF, A.; HAESEBROUCK, F. In vitro susceptibilities of *Mycoplasma hyopneumoniae* field isolates. **Antimicrobial Agents and Chemotherapy**, v.48, n.11, p.4470-4472, 2004.

VICCA, J.; MAES, D.; JONKER, L.; DE KRUIF, A.; HAESEBROUCK, F. The efficacy of tylosin premix for the treatment and control of *Mycoplasma hyopneumoniae* infections. **Veterinary Record**, v.156, p.606-610, 2005.

VILLARREAL, I.; MAES, D.; MEYNS, T.; GEBRUERS, F.; CALUS, D.; PASMANS, F.; HAESEBROUCK, F. Infection with a low virulent *Mycoplasma hyopneumoniae* isolate does not protect piglets against subsequent infection with a highly virulent *M. hyopneumoniae* isolate. **Vaccine**, v. 27, p.1875-9, 2009.

VRANCKX, K.; MAES, D.; CALUS, D.; VILLARREAL, I.; PASMANS, F.; HAESEBROUCK, F. Multiple-locus variable-number tandem-repeat analysis is a suitable tool for differentiation of *Mycoplasma hyopneumoniae* strains without cultivation. **Journal of Clinical Microbiology**, v.49, n.5, p.2020-2023, 2011.

WOOLEY, L.K.; FELL, S.A.; GONSALVES, J.R.; RAYMOND, B.B.; COLLINS, D.; KUIT, T.A.; WALKER, M.J.; DJORDJEVIC, S.P.; EAMENS, G.J.; JENKINS, C. Evaluation of recombinant *Mycoplasma hyopneumoniae* P97/P102 paralogs formulated with selected adjuvants as vaccines against mycoplasmal pneumonia in pigs. **Vaccine**, v.32, p.4333-4341, 2014.

YESKE, P.E. Economic impact of *M. hyopneumoniae* eliminations. In: **Proceedings of the XXIII IPVS Congress**, Cancun, p.336, 2014.

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